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A lov Story: A role for jim lovell in Drosophila neural development and fertility.

By

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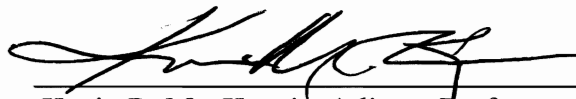
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## ABSTRACT

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A forward genetic screen looking for altered gravity response identified the gene jim lovell (*lov*). This gene is related to the gene fruitless, which specifies male courtship behavior in *Drosophila*. Like fruitless, *lov* encodes a protein with BTB-POZ domain and DNA binding domain, suggesting roles in transcription and ubiquitin-mediated degradation. In order to investigate the role(s) of *lov* further, several approaches have been taken. Five new mutations to the locus have been generated and their effects on various behaviors and development investigated. An antibody against the Lov protein has been generated and used to determine the wild type and mutant expression pattern of Lov during embryogenesis. The effects of ectopic expression and reduction of expression of *lov* in components of the nervous system was analyzed using the GAL4/ UAS system.

These studies establish that *lov* expression is limited to the nervous system beyond very early embryogenesis. In the wild type embryonic PNS, Lov is found in subsets of neurons of all three classes - External Sensory neurons, Chordotonal organs and Multiple Dendritic neurons suggesting that Lov plays a role in specifying the final identity of certain neurons. One of the mutants has reduced Lov expression in the embryonic nervous system.

Results from analysis of the new mutants and from the over/under-expression studies performed using the GAL4/UAS system have identified roles of *lov* in several behaviors and developmental processes. The altered expression of *lov* in the new mutant

alleles of *lov* results in decreased levels of male courtship directed towards a courtship partner as well as an increase in non-directed courtship. Additionally, various defects in sensory inputs were identified. The defects may contribute to in part to the reduced courtship. One of the mutants, *lov*<sup>66</sup>, has a male-male courtship phenotype. *lov*<sup>66</sup> also has a reduced fertility due to defects in spermatogenesis, egg formation, and in ability of females to use sperm effectively to fertilize eggs. Finally, both the over and under-expression of *lov* resulted in a reduction in courtship.

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## CHAPTER 1: INTRODUCTION

### Genes and Behavior

A fundamental question asked by both biologists and the general public is whether or not behaviors have a genetic basis. Disorders such as Autism and Alzheimer's appear to have a genetic basis, but the genes involved in these disorders have not yet been completely identified. Understanding the role of genes in these disorders will lead to better diagnosis and treatment of the disorders. In general, however, studying how genes affect human behavior is difficult. The hope is that by studying how genes affect *Drosophila* behavior, insight can be gained into their role in the human situation.

Behavior can be defined as an orderly, predictable pattern of activity that is produced by all members of a species (Baker et al., 2001). Behaviors can either be innate or modified by experience. Innate behaviors are ones in which there is a fixed pattern of activity and there is little to no variation within a species (Baker et al., 2001). Other behaviors are modified by experience. A member of a species may alter a behavior based on past experience or something in the environment.

There are two fundamental ways in which a gene can influence behavior. The first way is physiological. A gene may encode for a protein involved in the modification of a behavior and has an ongoing role in the animal (Baker et al., 2001). The white (w) gene is a good example of this. The white gene encodes for a transporter protein which is best known for transporting eye pigment to confer the wild type red eye color in *Drosophila*. This protein is also used to transport neurotransmitters. When white is mutated, alterations in normal behaviors such as courtship and gravitaxis occur (Armstrong et al. 2005).

The second way a gene may influence behavior is during development. A gene

may function to specify structures required for a particular behavior. These structures include the various appendages, such as legs and wings, required to carry out a behavior as well as the sensory organs required for input for a particular behavior. Finally, a gene can function during development by setting up the neural circuits in the CNS required for a given behavior (Baker et al., 2001).

Both the specification of sensory organs and appendages can influence multiple behaviors. Often, the sensory organs are used for inputs for many different behaviors and the appendages are used in generating different output responses. It is uncommon for a gene which helps to specify sensory organs or appendages to influence a single behavior. For example, wings are required for both courtship and flight. If a mutation disrupts normal wing formation, then it would be expected that both normal courtship and flight behaviors are disrupted.

In contrast, genes which specify neural circuitry most likely affect a single behavior or possibly a set of closely related behaviors. For several behaviors, regions of the CNS important for those behaviors have been identified. However, only for very few behaviors has the neural circuitry required for a particular behavior been identified (Baker et al., 2001).

These three types of specification are not independent of one another. Rather they function together to produce a behavior in the following fashion: sensory input → central processing → neural output → mechano/chemical output (Baker et al., 2001). Thus, determining how a gene influences a particular behavior involves identifying where in the behavior pathway a gene may function (sensory input, central processing, neural output, or mechano/chemical output) as well as its role in that part of the pathway.

## jim lovell and its role in Drosophila Behavior

The goal of the studies in this thesis is to understand the role(s) of the gene jim lovell (lov) with an emphasis on its roles in behavior. lov was originally identified in a screen looking for genes involved in gravitaxis. As shown here, it also has a role in courtship. These studies also show it is expressed throughout development as well as in adults, suggesting that lov may influence behavior either through developmental processes or have an ongoing role in behavior. Additionally, lov is expressed in both the central nervous system and the peripheral nervous system, suggesting that it may function in multiple steps of behavioral pathways.

## Gravitaxis

The force of gravity has had a large influence on life on Earth. Organisms have the form they do because of the constant downward force of gravity, and most multicellular organisms have developed organs specifically for sensing the gravity vector (Armstrong et al. 2005). In Drosophila, the organs important in gravity perception are stretch receptors called “chordotonal organs” found in the femur of the leg (femoral chordotonal organ) and in the antennae (the Johnston’s organ, the Drosophila organ of hearing) (Gopfert and Ito, 2009). An organism’s behavioral responses to gravity are known as gravitaxis. Unlike other senses, little is known about the molecular signaling pathways and central processing events that make up the response to gravity.

Gravitaxis in Drosophila was first studied by Hirsch and his collaborators in the 1960’s. They used a mass-assay maze apparatus to identify flies which had altered gravitactic responses. In the mass-assay maze, flies enter through a single entrance on one side of the maze and exit through one of nine exits at the other side. A light source

and yeast paste are placed on the exit side of the maze encourage the flies to move through the maze. The tubing is wide enough for the flies to walk, but not fly, through. At each junction, a fly has a choice of either up or down and the sum of its choices dictates which exit tube it uses to leave the maze (Figure 1-1). From a mix of wild-type populations, Hirsch and his collaborators isolated fly lines that showed altered (very high or very low) gravitaxic responses compared to the mixed wild-type progenitor population. The lines also showed stable inheritance of the altered gravitactic response, thus pointing to a genetic basis for this behavior (Erlenmeyer-Kimling and Hirsh, 1961; Hirsh 1959; Hirsch and Erlenmeyer-Kimling 1961; Hostetter and Hirsch 1967).

Additionally, micorarray studies done by Toma et al. (Toma et al. 2001) of the Hirsch high and low lines identified about 250 genes that had a two-fold or greater change in mRNA expression levels between the high and low lines. This identified new loci which may be important in gravitaxis. Among the genes identified in the studies were known circadian rhythm genes, cryptochrome (cry) and pigment-dispersing factor (Pdf). These two genes, along with a third gene, Pendulin (Pen) were chosen for further analysis. When mutants in these genes were tested in the maze, they demonstrated altered behaviors compared to wild type, thus confirming the results of the microarray (Toma et al. 2001). The finding that genes involved in circadian rhythm are also involved in another behavior can be attributed to the fact that circadian genes are known to regulate other genes in the nervous system (Toma et al. 2001).

Despite the strong evidence that gravitaxis has a genetic basis, no one had performed a large genetic screen to identify genes involved in gravitactic behavior. Therefore, the Beckingham lab performed a large forward genetic screen in *Drosophila* to

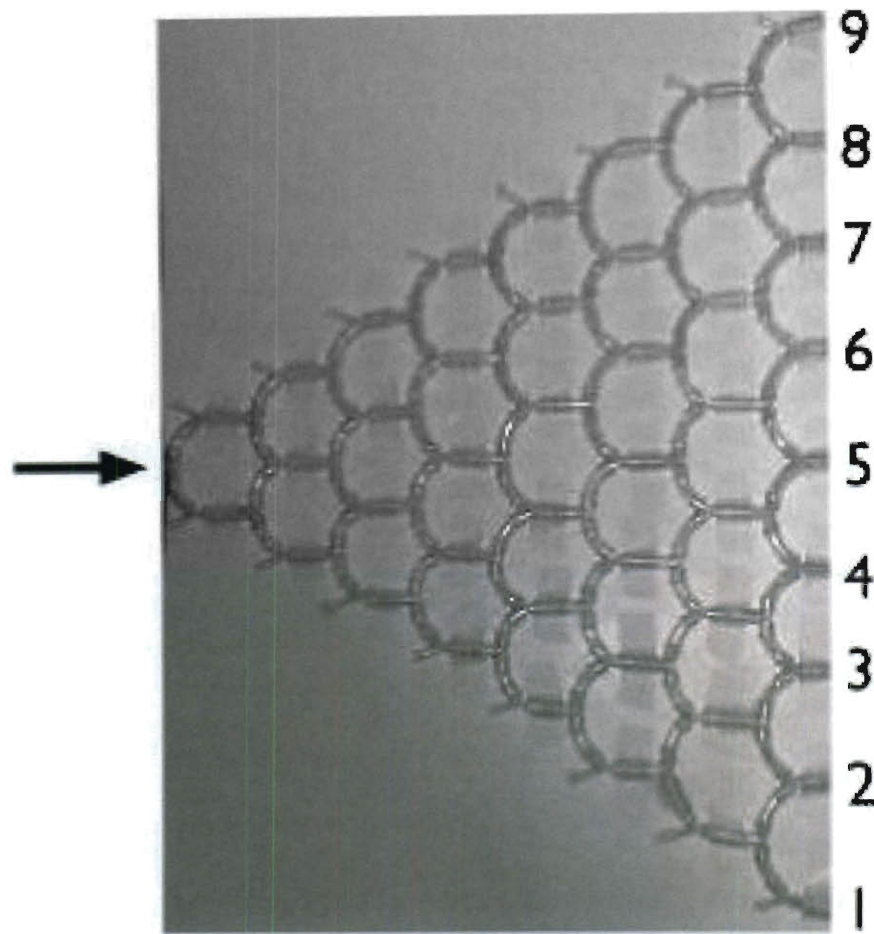


Figure 1-1: The Gravitax Maze. Photograph of a constructed maze. Flies enter through a single entrance (left, marked by the arrow) and exit through one of nine exits (right; exit 1 is at the bottom of the maze, 9 is at the top). A light source and yeast paste, placed on the exit side of the maze, encourage the flies to move through the maze. The tubing is wide enough for the flies to walk, but not fly, as they traverse the maze. At each junction, a fly has a choice of up or down (Adapted from Armstrong et al., 2005).

identify genes which appear to have a role in gravitaxis (Armstrong et al. 2005). They used a variation of the Hirsch Mass-Assay Apparatus (gravitaxis maze) to screen a collection of P{GawB} GAL4 enhancer trap lines generated previously (Yang et al. 1995).

The P{GawB} mutants were chosen because they have several advantages over other types of mutations (Beckingham et al. 2005). The exact location of the insertion in the genome can easily be identified through sequencing flanking regions. The defects produced by the insertion would be expected to be subtle since P-elements preferentially transpose into the upstream regions of the genes, altering the regulation of gene expression rather than disrupting protein coding sequences. The major advantage of this type of mutation, however, is the “enhancer-trapping” feature of the P-element. With the P-element inserted upstream of the coding region, the endogenous enhancer elements near the site of insertion will cause expression of the introduced GAL4 gene instead of the endogenous gene. The GAL4 protein, a transcription factor, can then activate expression of UAS-regulated transgenes (Figure 1-2). The system can be used to determine where endogenous gene expression is disrupted: the enhancer trap lines can be crossed with reporter lines carrying gene constructs such as UAS-lacZ (encoding  $\beta$ -galactosidase, which produces a blue precipitate when its colorless substrate is applied) or UAS-EYFP (encoding “enhanced yellow fluorescent protein”). Finally, the system can be used for functional analysis. For example, it can be used to drive expression of rescue constructs in the tissue that express GAL4.

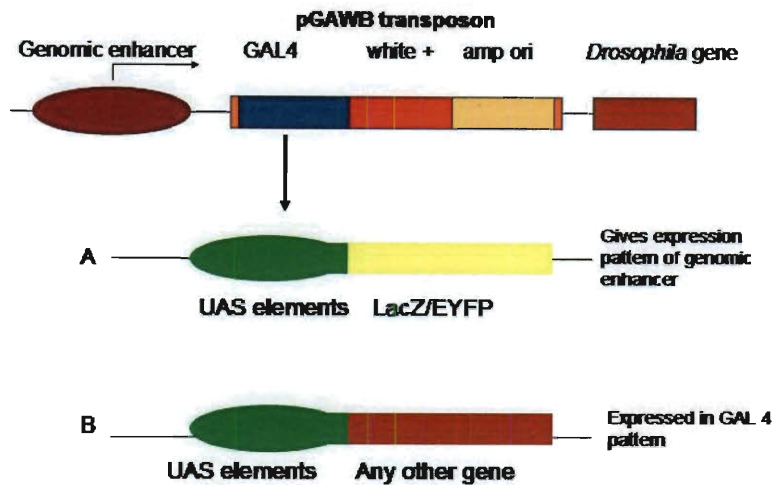


Figure 1-2: The GAL4 System. The GAL4 P-element transposons usually insert into the *Drosophila* genome, close to an enhancer element that would normally regulate an adjacent gene (shown in brown). The P-element contains three key components: the GAL4 gene (shown in blue), which will be expressed in a pattern dictated by the enhancer element, the white<sup>+</sup> gene (shown in red), a marker used to identify successful integration into the genome, and the bacterial plasmid sequences (shown in gold), amp<sup>R</sup> ori<sup>C</sup>, which allow for cloning of DNA adjacent to insertion site. Flies carrying the P{GawB} insertion are crossed to flies carrying a P-element insertion that has the bacterial  $\beta$ -galactosidase (lacZ) gene (or EYFP gene or any other gene of interest) linked to a promoter region carrying UAS sequences (typically of five of these GAL4 binding sites). Offspring of this cross will carry both constructs in their genome and will express lacZ (EYFP gene/gene of interest) in the pattern of GAL4. (Adapted from Beckingham et al., in 2005)

Behavior in the maze is dependent on many aspects of locomotor activity besides gravitaxis, therefore, in the screen performed by the Beckingham lab, additional behaviors were tested to control for defects in general locomotor function. The behavioral tests (climb, flight, and courtship) showed that the altered performance of the mutants in the maze assay was due to altered gravitaxis alone, rather than general defects in locomotion (Armstrong et al. 2005). The flies were also run through a horizontal maze to test if the flies' behavior in the vertical maze is a series of random choices or a directed choice due to the influence of gravity. The distribution of flies in the horizontal maze is close to a binomial curve, whereas the distribution of flies in the vertical maze is biased towards the upper exits (Figure 1-3). This shows that the behavior of the flies in the vertical maze is due to a directed choice influenced by gravity.

Three hundred lines were screened, and 23 of them were chosen for molecular analysis. The insertion site for each of these 23 lines was cloned and sequenced and the insertion site was determined using the published *Drosophila* genome sequence. Several of the genes identified have known roles in neural signaling or axon modeling in the organism. It appears that the insertion site for many of the lines affects only particular transcripts or regulatory elements of the adjacent gene (Armstrong et al., 2005). Therefore, it is possible that the mutations are affecting a subset of functions of the genes or a subset of tissues, and that the transcripts and regulatory elements affected are responsible for normal gravitactic behavior.

Eighteen of the genes identified by the screen have not been previously implicated in other behavioral responses besides gravitaxis. This number includes genes with both known and unknown functions (Table 1-1). The Beckingham lab has chosen to pursue



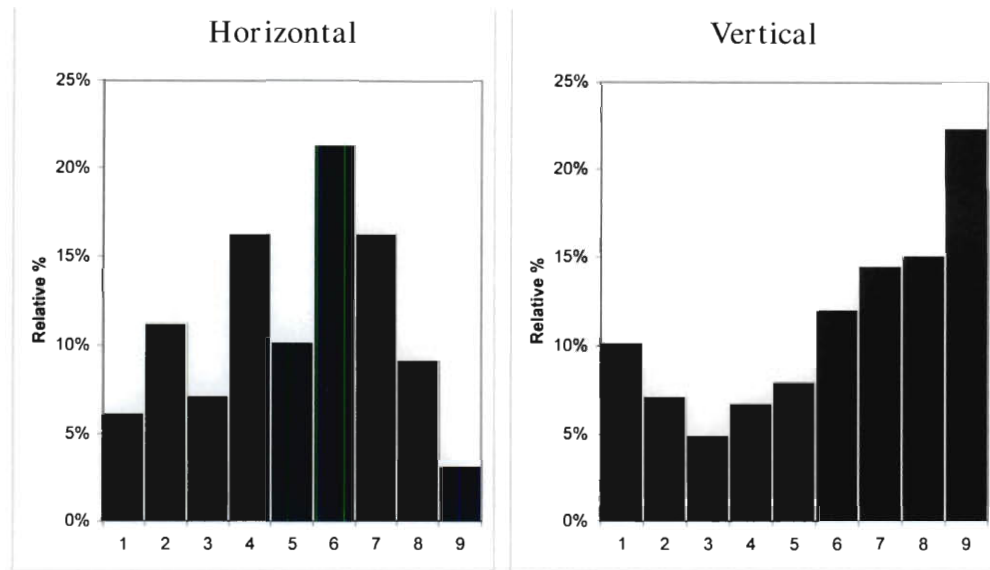


Figure 1-3: The gravitaxis maze assays responses to gravity. Horizontal (left) and vertical (right) maze profiles for wild-type flies. Horizontal exits are numbered 1 (left) to 9 (right) as viewed from entrance of the maze and vertical exits are numbered 1 (bottom) and 9 (top). The distribution of flies in the horizontal maze is close to a binomial curve, whereas the distribution of flies in the vertical maze is biased towards the upper exits. (Adapted from Armstrong et al., 2005)

Table 1-1: Insertion sites for the P{GawB} gravitaxic mutations

Line	Function of candidate gene(s)	Protein homologs
22Y	br: BTB/POZ-domain and C2H2- zinc-finger-containing transcription factor affecting salivary gland, ovary, adult brain and eye	Caenorhabditis elegans mouse, human
82Y	otk: receptor tyrosine kinase involved in neural pathfinding and cell adhesion	Mammals and plants
91Y	Tkr: a BTB/POZ-domain DNA- and actin-binding proteins	Other insects
93Y	w: ATP-binding cassette transmembrane pigment precursor transporter	Fungi, insects, vertebrates
144Y	CG6330: putative uridine/purine phosphorylase	C. elegans, mouse and human
161Y (FBti0004614)		
179Y (FBti0004612) (BL-3733)	CG11940: putative PH- and RA-domain-containing homologs of Grb molecular adaptors	C. elegans MIG-10; human ALS2CR9 (familial ALS); mouse and human GRB14, GRB-10, GRB-7
181Y (FBti0004611)	CG10543: putative C2H2 zinc- finger transcription factor	Yeast, C. elegans, vertebrates
187Y (FBti0004608)	(see 144Y)	C. elegans, mouse, human
202Y	CG17090: putative S/T kinase with possible roles in cell proliferation, ectoderm development, neurogenesis and apoptosis	Fungi, plants, C. elegans, vertebrates
227Y	CG3857: P-loop-containing NTP hydrolase	Insects, C. elegans, fish, chicken, mouse, human
248Y	dlg1: guanylate kinase containing PDZ, SH3 and P-loop domains, with roles in septate junction formation and synapse structure	C. elegans, fish, chicken, mouse, rat, human (channel- associated protien of synapse 'chapsyn-110')
c65	Hr38: ligand-dependent nuclear receptor with C4 zinc finger, with roles in cuticle and joint formation	Animals
c105 (FBti0018459)	CG32598: putative gene similar to basic transcription factors	C. elegans, mouse, humans: basic transcription factor 3
c189	CG15507: putative gene of unknown function; very Q-rich kay: JNK-cascade-activated bZIP transcription factor with roles in dorsal closure and ectoderm development	none C. elegans, vertebrates (C-FOS)
c263	yuri:	Other Drosophila species
c282	CG3967: putative acyl-coA N- acetyltransferase Shc: signal-transduction adapte protein with SH2, PH-like, and phosphotyrosine-binding domains downstream of EGFR tyrosine kinases, with roles in Torso signaling and neural impulse transmission	C. elegans, mouse, human C. elegans, fish, chicken, mouse, rat, human
c287a	esg: C2H2 zinc-fingertranscription factor involved in development of peripheral nervous system and tracheal system	Yeast, invertebrates, vertebrates (in humans, SLUG)
c305a	Cka: WD-40 (protein-protein interaction)-domain-containing part of JNK cascade involved in dorsal closure and cuticle development	Yeast, plants, C. elegansinsects; mouse striatin; human cell-cycle autoantigen SG2NA, striatin, zinedin
c522 (FBti0004519) (BL-3747)	CG32423: putative RNA- binding domains protein with RNP-1 and RBD domains	Insects, Xenopus, fish, human (Scr3) mouse (Rbms2, Scr3) andhuman (Scr3)
c609 (FBti0004496)	CG32100: putative gene of unknown function	Mosquito
G6	CG5017: putative nucleosome assembly chaperone	Anopheles mosquito, Xenopus, fish, chicken, mouse, rat, human
G8	CG7832: gene of unknown function whose overexpression causes extra wing veins and rough eyes	

\*Genes bolded have been further studied in the Beckingham Lab. (Adapted from

Beckingham et al., in 2005)

further the genes which appear to have novel functions and have related genes in higher organisms, one of the genes being *jim lovell* (*lov*), the subject of this thesis.

#### Overview of Courtship Behavior in *Drosophila*

Of all the innate behaviors displayed by *Drosophila*, male courtship behavior is probably the most studied. Courtship behavior consists of six consecutive steps (Figure 1-4) (Greenspan, 1995; Greenspan, 2000; Hall, 1994). Typically, a male will perform the individual steps multiple times before continuing on to the next step (Bray and Amrein, 2003). The first step involves the male orientating towards the female. Next, the male taps the female's abdomen with his forelegs. In tapping the female, the male is "tasting" the female to determine if she is a suitable mate. Recently mated females will have a different "taste" than virgin females. Third, the male will extend one wing and vibrate the wing to "sing" a courtship song. The rate of vibration is different for different species of *Drosophila*. The female uses this song as a way to ensure that her prospective mate belongs to the correct species. Next, the male licks the female's genitalia again to "taste" the female and ensure she is a suitable partner. Finally, the male will make multiple copulation attempts before successfully copulating with the female. If a female is receptive to the male's copulation attempts, she will allow him to copulate by spreading her wings, allowing access to her genitals. If the female is unreceptive to the copulation attempts, she will prevent him from copulating by walking away, flicking her wings, kicking her hind legs, and extending her ovipositor (Bray and Amrien 2003).

Each step of courtship requires a number of sensory inputs for the process to progress. Additionally, proper neural processing and output (neural, mechanical, and chemical) are required for normal courtship (Figure 1-5). Orientation involves visual

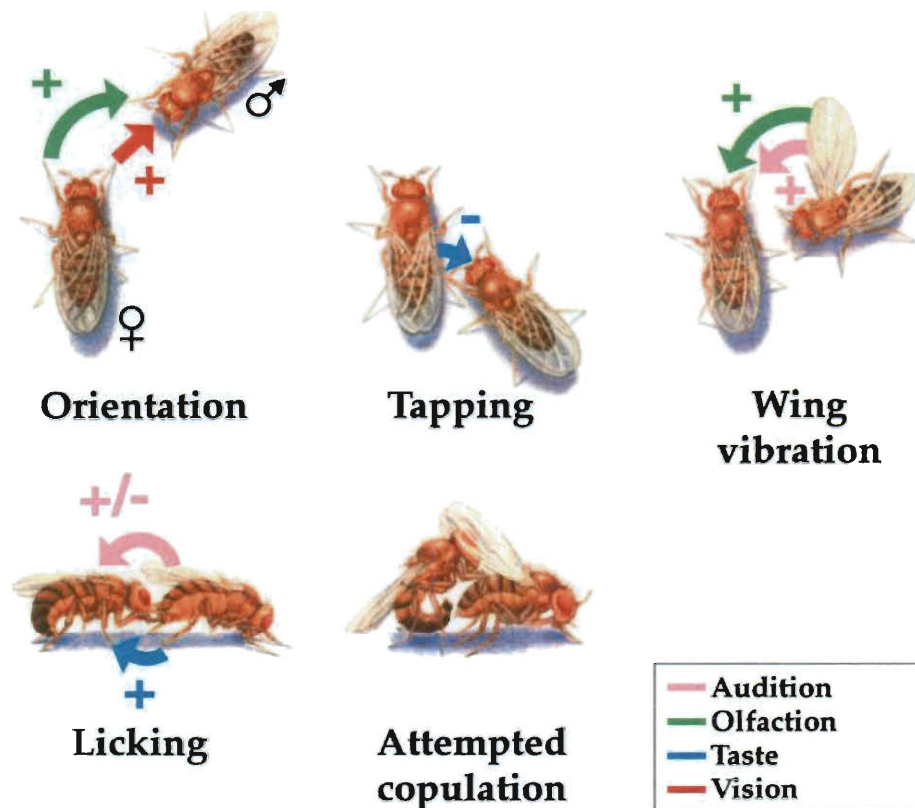
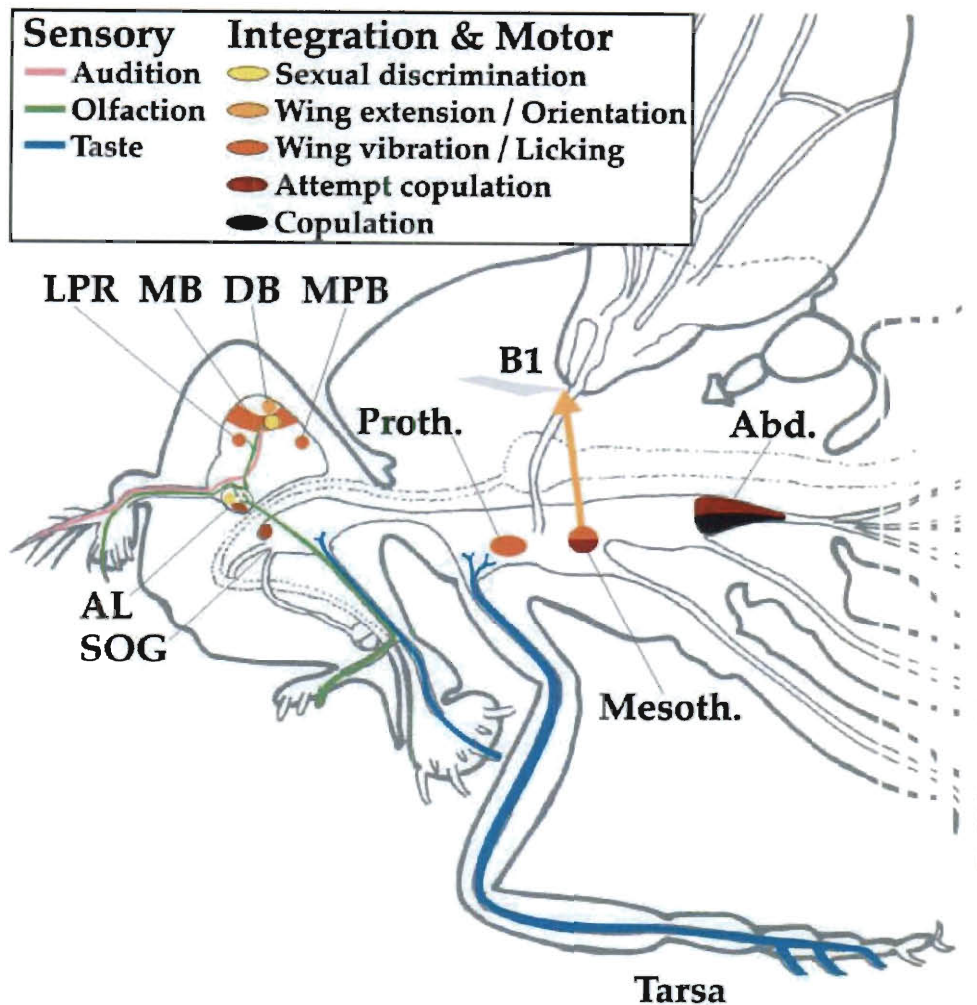


Figure 1-4: *Drosophila* Courtship. First, the male orientates towards the female. Second, the male taps the female's abdomen with his forelegs. Third, the male will extend one wing and vibrate the wing to "sing" a courtship song. Fourth, the male licks the female's genitalia again to "taste" the female. Finally, the male will make multiple copulation attempts before successfully copulating with the female. (Adapted from Greenspan 1995)



Key: abdominal ganglion (Abd.), antennal lobes (AL), B1 direct flight muscle (B1), dorsal brain (DB), lateral protocerebrum (LPR), mushroom bodies (MB), mesothoracic ganglion (Mesoth.), medial posterior brain (MPB), prothoracic ganglion (Proth.) and subesophageal ganglion (SOG)

Figure 1-5: Location of Neurons Involved in Courtship. Orientation involves visual input for the male to recognize the female. Both tapping and licking require chemosensory input to ensure that the female is a suitable courtship partner. Auditory reception is required for the female to hear the courtship song that a male produces. Finally, wing extension, attempted copulation, and copulation all require locomotor output to occur. Proper processing and neural output is also required for courtship to occur. (Adapted from Greenspan 1995)

input for the male to recognize the female. Both tapping and licking require chemosensory input to ensure that the female is a suitable courtship partner. Auditory reception is required for the female to hear the courtship song that a male produces. Finally, wing extension, attempted copulation, and copulation all require locomotor output to occur (Bray and Amrein, 2003). A number of genes are now known to have roles in these various aspects of courtship and many of the genes are regulated by the genes in the sex determination hierarchy.

Somatic sexual development and sexual behavior in *Drosophila* are controlled by the same set regulatory genes. The sex determination hierarchy involves a series of alternative splicing events and is dependent on the ratio of X chromosomes to autosomes (Figure 1-6) (Cline and Meyer, 1996). In females, the ratio is 2:2 X chromosomes: autosomes. This ratio results in a functional form of the protein encoded by the master regulatory Sex lethal (Sxl) being produced. Sxl is a splicing regulatory protein which in turn splices the mRNA of a transformer (tra) into a female-specific functional form. Tra, like Sxl, is a splicing regulatory protein and together with Tra2 (yet another splicing regulatory protein) functions to control female specific splicing of transcripts from double-sex (dsx) and fruitless (fru). A female-specific form of the Dsx protein is produced but female-specific splice variants do not produce a functional Fru protein (Usui-Aoki et al 2000). In males, the ratio of X chromosomes to autosomes is 1:2. In this case, a non-functional form of Sxl is produced. As a result, no functional form of Tra is produced. By default, the male-specific splicing of fru and dsx occurs producing functional male-specific forms of Dsx and Fru protein. Dsx is responsible for correct male morphology whereas Fru is responsible for normal male courtship (Baker et al., 2001).



Figure 1-6: The Sex Determination Hierarchy. The sex determination hierarchy is a series of alternative splicing events which is dependent on the ratio of X chromosomes to autosomes. In females, the ratio is 2:2 X chromosomes:autosomes and a functional form of the master regulator Sex Lethal (Sxl) is produced. In turn, Sxl splices the mRNA of transformer (tra) into a female-specific functional form. Tra (a splicing regulatory protein) and Tra2 (another splicing regulatory protein) then generate female-specific splice forms of double-sex (dsx) and fruitless (fru) transcripts. A female-specific form of Dsx protein is produced. In males, the ratio of X chromosomes:autosomes is 1:2. In this case, a non-functional form of Sxl is produced. As a result, no functional form of Tra is produced. By default, the male forms of the Fru and Dsx proteins are produced (Dauwalder 2008).

As can be seen from Figure 1-7, the *fru* gene has four promoters, P1-P4 (Ito 1996; Ryner 1996). Transcripts from all four promoters encode a protein with a BTB domain. The transcripts from P2, P3, and P4 are non-sex-specific and appear to be responsible for vital functions that are separate from the products of the P1 promoter (Baker et al., 2001). Only the sex-specific transcripts of *fru* are part of the sex determination hierarchy. The sex-specific forms of *fru* result from alternative splicing of transcripts from the P1 promoter (Goodwin et al., 2000; Baker et al., 2001) (Figure 1-7). For both males and females, there are three transcripts which differ at the 3' end by using different exons that encode alternative zinc fingers. At the 5' end, male and female products differ by the addition of 101 amino acids to the male specific products (Goodwin et al., 2000; Ito 1996; Ryner 1996). A functional form of female-specific Fru is not produced due to the female-specific splicing of *fru* which places a stop codon very early in the transcript. As previously mentioned, courtship is a sequence of behaviors in which each step is dependent on execution of the previous step. Mutant analysis of various *fru* mutants demonstrates that *fru* has a role in each step of courtship rather than being required only for the first step of courtship (Goodwin et al., 2000; Baker et al., 2001). Several of these mutants are unable to discriminate males from females. Other mutants are unable to produce a courtship song or produce an incorrect courtship song. Finally, many of the mutants do not attempt to copulate (Ryner, 1996; Ito, 1996; Villella et al. 1997; Goodwin et al., 2000) (Table 1-2). These defects are not due to general defects in behavior as both locomotor and wing usage behaviors are wild type (Villella et al, 1997). Examination of the expression pattern of the male-specific isoforms provides insight into how *fru* may



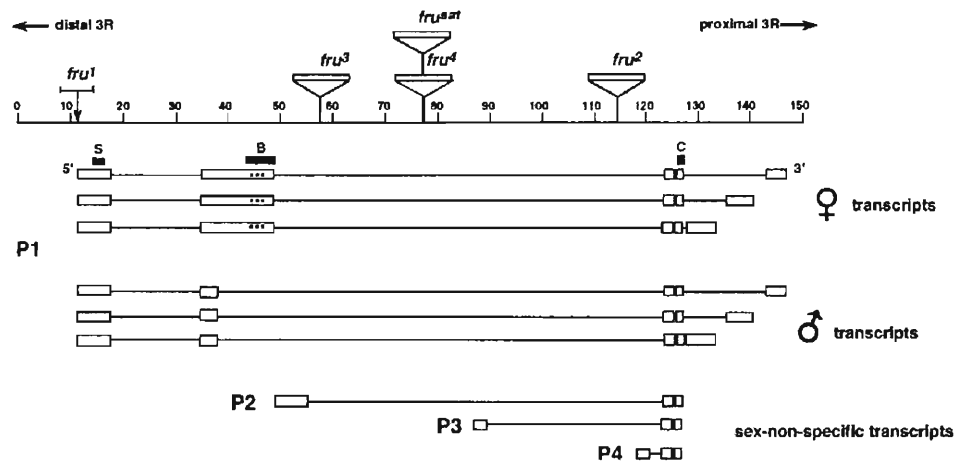


Figure 1-7: Genomic Structure of *fru*. *fru* has four promoters (P1-P4) and transcripts from all four promoters encode a protein with a BTB domain. The transcripts from P2, P3, and P4 are non-sex specific and appear to be responsible for vital functions that are separate from the products of the P1 promoter. The sex-specific forms of *fru* are due to alternative splicing of transcripts from the most distal promoter, P1. In both males and females, there are three transcripts which differ at the 3' end by using different exons that encode for alternative zinc fingers. At the 5' end, these male and female transcripts products differ by the addition of 101 amino acids to the male specific products. (Adapted from Goodwin et al., 2000)

Table 1-2 Courtship Defects of the fru Mutants

fru Mutation	Courtship Defects	Source
fru <sup>1</sup>	Unable to discriminate males from females; Abnormal courtship song; No copulation attempts	Ryner 1996; Villella 1997; Goodwin et al., 2000
fru <sup>2</sup>	Unable to discriminate males from females; Abnormal courtship song	Ryner 1996; Villella 1997; Wheeler et al. 1998; Goodwin et al., 2000
fru <sup>3</sup>	Wing extension but does not produce courtship song; No copulation attempts	Ryner 1996; Villella 1997; Goodwin et al., 2000
fru <sup>4</sup>	Unable to discriminate males from females; Wing extension but does not produce courtship song; No copulation attempts	Ryner 1996; Villella 1997; Goodwin et al., 2000
fru <sup>sat</sup>	Male-male courtship; No copulation attempts	Ito et al. 1996; Yamamoto 1996; Yamamoto 1997; Yamamoto 1998; Goodwin et al., 2000

regulate courtship. Expression of the male-specific fru product is primarily in the CNS (Ryner 1996, Goodwin et al., 2000, Lee et al. 2000) and begins in the later stages of development and continues into adulthood. Expression begins at the end of larval stage and expression levels continue to rise until they peak two days into pupal life. After this point, fru expression decreases and by the end of pupal life, only about 500 cells are expressing fru (Baker et al., 2001).

fru expressing neurons are found throughout the CNS in about 20 small clusters. Additionally, there are single cells expressing fru throughout the brain and ventral nerve cord (Lee et al. 2000). FruM is expressed in higher order neurophils rather than sensory or motor neurons. The morphology of the fru expressing cells suggests that they are either local circuit neurons or interganglionic interneurons (Baker et al., 2000). Based on altered expression in fru mutants, it is believed that the fru expressing neurons receive information from mainly non-sex specific neurons, process that information in a sex-specific manner, and generate an output that is executed by non-sex specific motor neurons (Goodwin et al., 2000; Lee and Hall, 2001). It would appear then that fru sets up the required neural circuitry in the CNS required for normal courtship.

Together fru and dsx regulate the expression other genes involved in courtship. For some genes, either fru or dsx is required, but for others expression of both genes is required for proper regulation. Microarray analysis has been use to identify genes which are downstream of either fru or dsx (Goldman and Arbeitman 2007). The genes identified were expressed in a variety of tissues, including some tissues, such as the fat body, which had not previously been identified as important for courtship.

One such gene is takeout (to), which was found to have higher expression in male

heads (Dauwalder et al. 2002). Mutants in *to* have reduced courtship due to reduced levels of initiation and duration of courtship. However, they are able to perform all the steps of courtship (Dauwalder 2008). *to* was found to be expressed in the fat body which surrounds the brain. Experiments in which the male fat body was feminized caused a reduction in courtship suggesting that the fat body has an important role in courtship (Lazareva et al. 2007). Both *Dsx* and *FruM* are required for full activation of *to* (Dauwalder et al. 2002). Potential *Dsx* consensus binding sites are found within 1kb upstream of the *to* transcription start site and *Dsx* has been shown to be expressed in the fat body (Erdman et al. 1996; Dauwalder, 2008). Therefore, *Dsx* may be acting directly to regulate expression of *to*. *FruM* expression is not found in the fat body, suggesting that *FruM* may act indirectly either through the generation of circulating signals or possibly other effects modulated by the *FruM* expressing cells (Lee et al. 2000; Usui-Aoki et al. 2000; Goodwin et al. 2001; Dauwalder, 2008). Based on experiments in which *to* and *fru* mutants are combined, *to* may be functioning as a secreted protein and having a physiological role rather than a developmental role, but its exact function is unknown (Dauwalder, 2008).

Another gene that was identified as being regulated by *fru*, *dsx*, or both, is defective proboscis extension response (*dpr*) (Goldman and Arbeitman, 2007). Microarray analysis revealed that *dpr* expression is higher in wild type males than in *fru* P1 mutants, suggesting that *dpr* is regulated by *FruM*. Additionally, the expression patterns of *dpr* and *fruM* overlap in CNS regions known to be important for courtship. *dpr* expression is also found in the PNS in regions important in courtship, such as the foreleg and the proboscis (Goldman and Arbeitman 2007). Therefore, *dpr* may play a

role for in primary sense response and higher order processing. Finally, both *dpr* mutants and reduced *fru* P1 expression in *dpr* expressing cells (which reduces *dpr* expression) result in reduced courtship latency and reduced time to attempted copulation, suggesting that *dpr* helps to regulate the timing of different stages of courtship (Goldman and Arbeitman 2007).

An example of a gene which is regulated by *dsx* alone is *Gr68a*. *Gr68a* is a pheromone receptor that is only expressed in neurons of about ten male specific taste bristles in the foreleg. Reduced courtship was observed when either the *Gr68a* expressing neurons were inactivated or when *Gr68a* expression was reduced through the use of RNAi. Furthermore, when *Gr68a* expression was reduced through the use of RNAi, males appeared to stall in the second stage of courtship-tapping. However, overall sensory perception required for two other related behaviors, sugar recognition and gravity locomotion was not affected in males lacking functional *Gr68a* expressing neurons. This would suggest that *Gr68a* functions in pheromone perception and that *Gr68a* essential's role in courtship is recognizing female pheromones (Bray and Amrein, 2003).

The genes discussed above demonstrate ways in which genes can function to regulate complex behaviors. They also demonstrate how different genes interact to generate these complex behaviors. Furthermore, *dpr* demonstrates that a gene can function in both sensory input as well as neural processing to help modulate behavior.

jim lovell

One line identified in the Beckingham gravitactic screen was 91Y. Flies in this line exited the maze through the bottom exits, making it a “low line” (Figure 1-8). Because behavior in the maze is dependent on many aspects of locomotor activity,

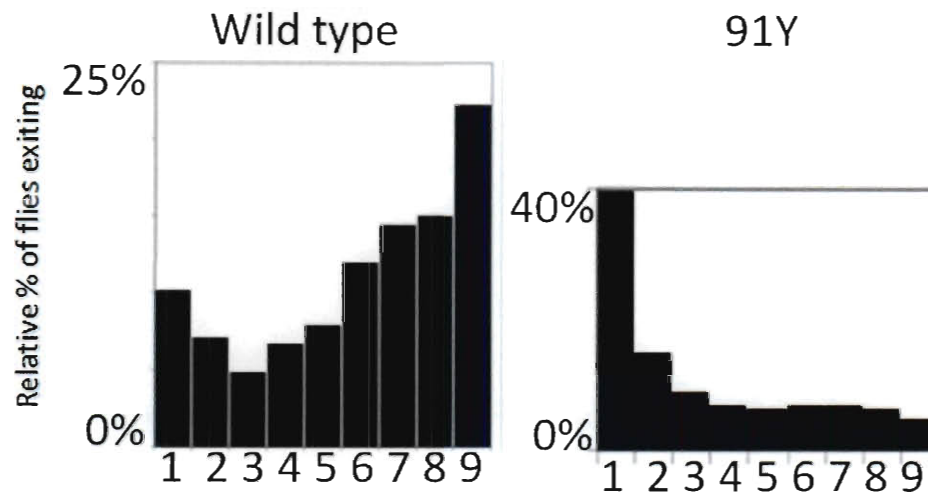


Figure 1-8: Gravitaxis Maze Results for Control and lov 91Y Insertion Flies. In control flies, the majority of the flies exit the gravitactic maze through the upper exits. In 91Y, the majority of the flies exit the gravitactic maze through the bottom exits (Adapted from Armstrong et al., 2005).

additional behaviors were tested to control for defects in general locomotor function. Other indicators of locomotor behavior, such as the Courtship Index, the Flight Index, and climbing, were normal (data not shown). Thus, the mutation specifically affects gravity responses (Armstrong et al., 2005).

91Y is inserted into a previously uncharacterized gene we named jim lovell (lov) (Figure 1-9). Based on sequence homology lov is a putative transcription factor, with four predicted transcripts based on cDNA sequencing (BDGP 2000). All four transcripts are believed to encode the same protein.

The protein is predicted to contain three conserved domains: Helix-turn-Helix Pipsqueak (Psq) and Helix-turn-Helix Pipsqueak-like domains (both located in the third common exon), which are involved in DNA binding, and a BTB (Broad- Complex, Tramtrack, and Bric-a-brac)/POZ (poxvirus and zinc) domain (located in the first common exon), which is involved in protein- protein interactions and possibly ubiquitin-mediated degradation. These domains are evolutionarily conserved from *Drosophila* to humans. The Helix-turn-Helix Pipsqueak (Psq) and Helix-turn-Helix Pipsqueak-like domains are essential for Target-of-Polycomb Group complex regulation, which is required for maintaining the repressed chromatin state of some developmental regulatory genes (Lehmann et al., 1998; Siegmund and Lehmann, 2002; Strutt and Paro, 1997). The other domain, BTB/POZ, mediates homomeric and heteromeric dimerization. The BTB/POZ domain is found near the N-terminus of some zinc finger proteins, and the POZ domain mediates transcriptional repression and interacts with components of the histone deacetylase co-repressor complex (Zollman et al., 1994; Couderc et al., 2002).

Recent studies indicate a new role for the BTB/POZ domain in ubiquitin-

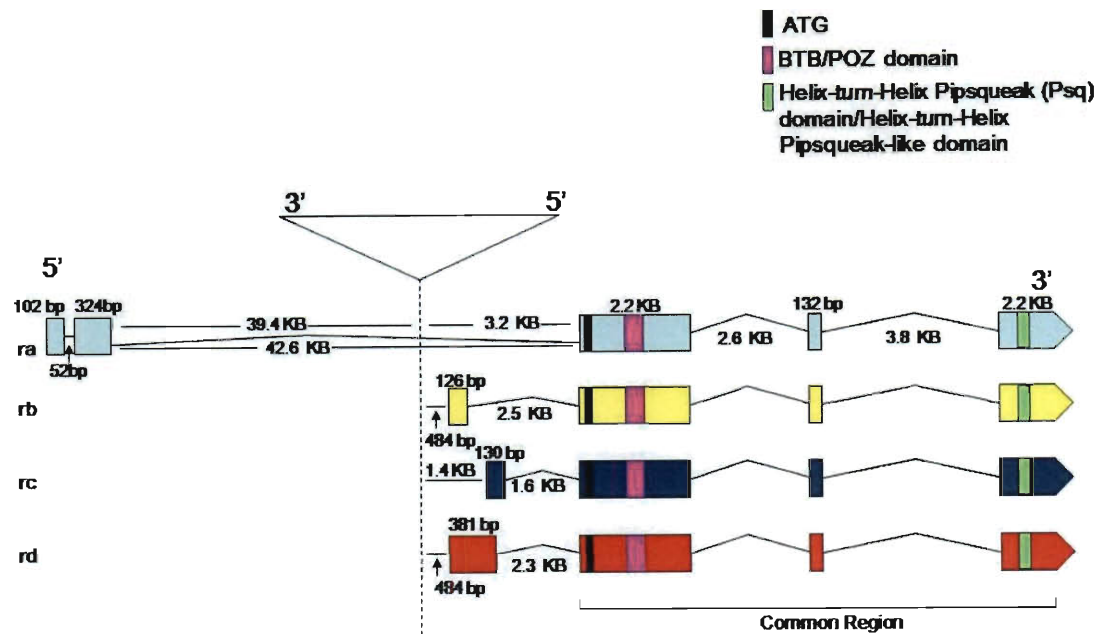


Figure 1-9: Transcripts from the *lov* gene and the position of the 91Y Transposon.

There are four predicted transcripts for *lov* (Flybase.net): RA (4731 nt), RB (4431 nt), RC (4435 nt), and RD (4685 nt). RA, RB, RC, and RD are translated into a single protein that is predicted to be 1046 amino acids long. The 91Y transposon is inserted 465 bases 5' of the transcription start site of *lov*-RB and RD; 1406 bp 5' of *lov*-RC; ~37 kb in ~40 kb intron (5' UTR) of *lov*-RA start (Armstrong et al., 2005).



mediated degradation. In *C. elegans*, it was found that a BTB/POZ-containing protein, MEL26, interacts with CUL 3 ubiquitin ligases through its BTB/POZ domain. MEL26 was also found to bind the substrate to be degraded, thus combining the roles of Skp1 and F-box proteins in the SCF complex model of ubiquitin degradation (Figure 1-10) (van den Heuvel, 2004). Similar roles have been found for the BTB/POZ domain proteins in *Arabidopsis* and *S. pombe* (Gingerich et al. 2005; Geyer et al. 2003). Thus, the same may hold true in *Drosophila*, but this function of the BTB domain has yet to be studied in *Drosophila*.

Recent studies have also found a link between ubiquitin modification and the development and function of synapses in the nervous system. Evidence suggests that ubiquitin and ubiquitination enzymes may be important regulators of the development, function, and plasticity of synaptic connections (DiAntonio and Hicke, 2004). For example, DiAntonio et al. (2001) found that ubiquitination is a key regulator of synapse formation and growth at the *Drosophila* neuromuscular junction. They found that when the de-ubiquitinating enzyme fat facets (faf) is overexpressed in the developing nervous system, there is overgrowth of synapses and impaired synaptic transmission (DiAntonio et al. 2001, DiAntonio and Hicke, 2004). These two sets of recent findings, taken together, suggest that lov may have a role in ubiquitin degradation and synaptic remodeling.

In *Drosophila*, there are approximately 60 genes which encode proteins that contain the BTB domain, nearly all of which also encode zinc finger domains. The presence of the zinc finger domain suggests that these genes encode transcription factors. Like lov, several of these genes encode multiple protein isoforms of the same protein due

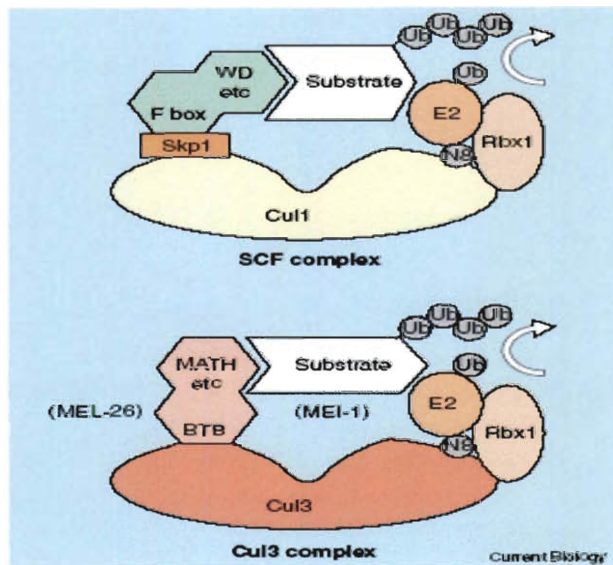


Figure 1-10: Comparison Between the SCF Complex and Cul3 Complex. In the SCF complex, two proteins, Skp1 and F box, are used to connect the ubiquitin ligase and the substrate to be degraded. In the CUL3 complex, the BTB/POZ domain containing protein (in this case MEL26) was found to bind both the ubiquitin ligase and the substrate to be degraded (Adapted from van den Heuvel, 2004).

to alternative splicing and multiple promoters. Many of these genes function in the nervous system where they function in synapse specificity, pathfinding, and adult brain morphogenesis (Gorczyca et al. 1999; Seeger et al. 1993; Restifo and Hauglum, 1998). The most studied of these genes is *fru*. As mentioned above, *fru* is involved in *Drosophila* courtship behavior, and mutations in male-specific transcripts results in males courting other males. It has been suggested that other BTB containing genes, such as *lov*, have behavioral roles, with individual transcripts regulating individual behaviors (Baker et al., 2001).

Examination of the expression pattern of the *lov* also provides insight into the potential functions of *lov*. Based on microarray data, the tissues which have the highest levels of predicted *lov* expression are the testes and the head (Flyatlas.org). A high level of expression of *lov* in the testes suggests that *lov* may have a role in spermatogenesis. The high expression level in the head suggests that *lov* may function in the CNS to regulate behavior. Finally, the Crews lab has found that *lov* mRNA is expressed in specific pattern in the ventral unpaired median (VUM) neurons (a type of motor neuron) in the CNS midline cells through in situs during midway through development and in the ventral nerve cord from there on out (Kerney et al. 2004).

#### General Outline of the Thesis Studies

The studies presented here were aimed at exploring further the role of *lov* in the nervous system and in behavioral responses with a particular emphasis in determining whether *lov* influences courtship behavior. First, I will show that in the original *lov* mutant, 91Y, normal *lov* expression is likely disrupted in tissues believed to be important in gravitaxis and courtship. Next, I will discuss the generation of new mutants in *lov* and

their molecular characterization. Third, I will discuss the generation of an antibody against Lov and its use to characterize the expression pattern in the embryonic nervous system. Next, I will examine one mutant, *lov*<sup>66</sup>, and its defects in fertility, analyzing both the developmental and behavioral aspects. I will then discuss courtship defects that were observed by examining all the *lov* mutants. I have also examined other behaviors in these mutants to help determine the underlying cause of the observed courtship defect. Next, I will discuss the generation of *lov* transgenic lines which were used to further examine the role of *lov* in development, neural function, and courtship. Finally, I will discuss an attempt to identify interacting partners of *lov* through the use of co-immunoprecipitation and mass spectrometry.

## CHAPTER 2: EXAMINATION OF 91Y-EYFP EXPRESSION

Examination of the 91Y-EYFP expression pattern provides insight into where *lov* is normally expressed and suggests possible functions of *lov*.

The original P{GawB} mutants that were screened in the gravitaxic maze were chosen because they have advantages over other types of mutations, with the major one being the “enhancer-trapping” feature of the P-element (Beckingham et al. 2005). With the P-element inserted upstream of the coding region, the endogenous enhancer elements near the site of insertion will express the introduced GAL4 gene in the pattern of the endogenous gene. Often, only specific subsets of the enhancers are affected. The resulting disruption of the normal endogenous gene expression may alter physiology and/or development in the affected tissues. The GAL4 protein, which is a transcription factor, can be used to activate expression of UAS-regulated transgenes (Figure 1-2) and by this route, it can be determined where endogenous gene expression is potentially disrupted by the P-element. This is accomplished by crossing the P{GawB} enhancer trap line to a reporter line carrying the gene construct UAS-EYFP (encoding “enhanced yellow fluorescent protein”). The EYFP expression pattern in the progeny then reveals the tissues expressing GAL4 and thus the possible tissue of origin for any mutant phenotype produced by the P{GawB} mutant.

Previously, the 91Y GAL4 expression pattern in the brain has been characterized (Fly-trap.org). GAL4 expression in the brain is complex with widespread expression throughout. To gain insight into where else *lov* is normally expressed, individuals from 91Y P{GawB} line were crossed with individuals homozygous for a UAS-EYFP insertion. EYFP expression in the offspring was visualized with a fluorescence

microscope. EYFP expression was found in three locations in the adult (Figures 2-2 and 2-3). First, in a small number of animals, expression was found in the stretch receptor of the wing. The function of this receptor is unknown, but may provide that animal information about wing location in a non-flying state.

The second location is in the leg chordotonal organ, which is believed to be important in gravitaxis responses. Eleven complete leg sets were examined in males and ten complete leg sets were examined in females. For both males and females, this expression pattern was observed in all individuals examined (Table 2-1).

Lastly, EYFP expression was found in the tarsus, the most distal segment of the leg. However, this expression was limited to the front leg of males only. Of 11 sets of legs examined for males and 10 sets of legs examined for females, 19 out of 21 male front legs had EYFP expression. In contrast, none of the male middle or hind legs and none of the female legs had EYFP expression (Table 2-1). This is the first indication that *lov* has a sex-specific expression pattern and suggests the presence of a male specific enhancer element.

In larvae, EYFP expression was limited to a small number of cells in the leg imaginal disc at the position of the developing chordotonal organ (Figure 2-4). It seems likely that these are the developing neurons of the adult leg chordotonal organ in which EYFP expression was found, suggesting that *lov* expression begins soon after the formation of these neurons.

What does the EYFP expression in 91Y say about the possible function of *lov*?

The limited expression pattern observed in 91Y provides much insight into the potential functions of *lov*. Chordotonal organs function as proprioceptors. Thus, the

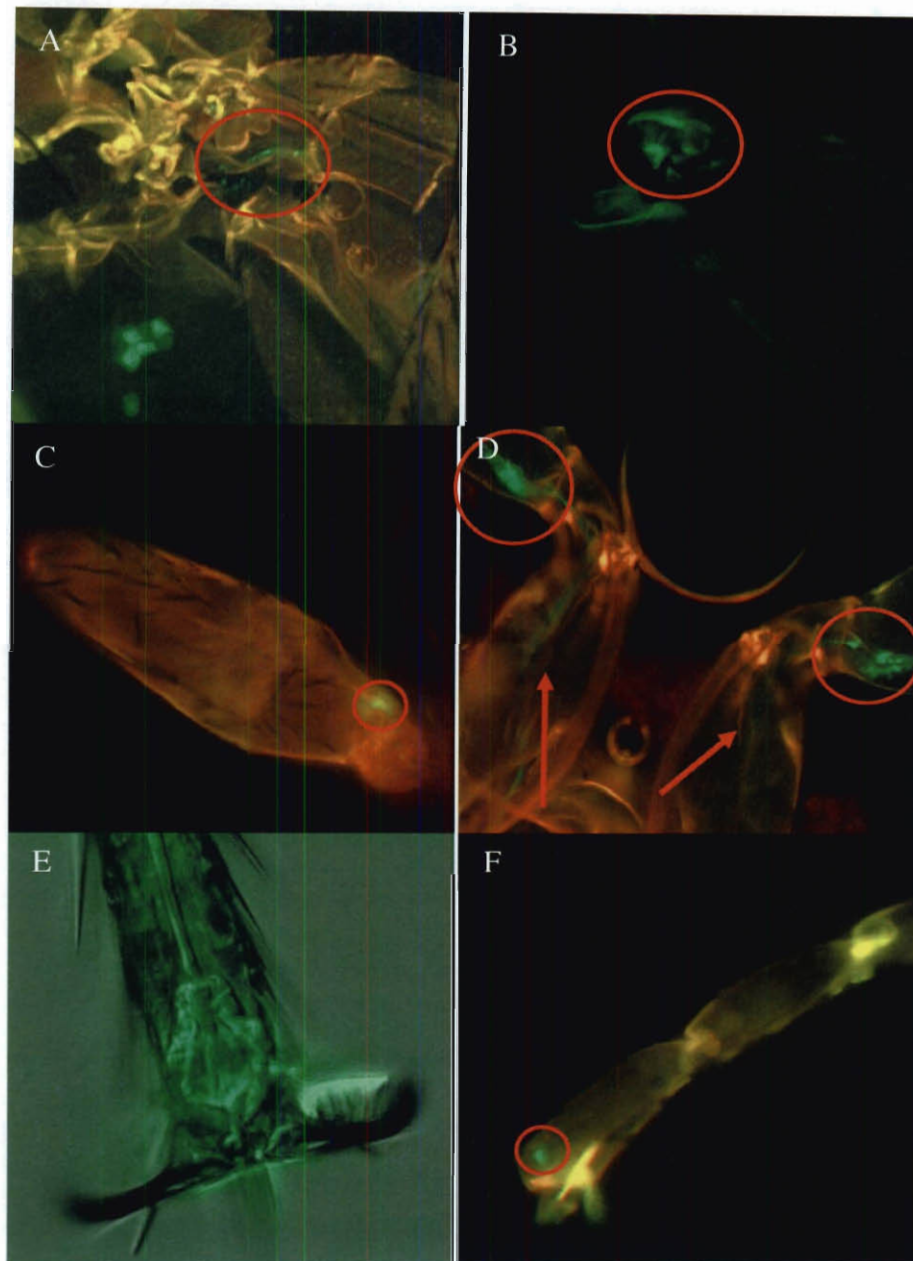


Figure 2-1: EYFP expression in adults under the 91Y GAL4 driver. A&B. Stretch Receptor of the Wing. In a limited number of animals, EYFP expression is observed in the stretch receptor of the wing. C&D. Leg chordotonal organ (CHO). EYFP expression is observed in the CHO of all three pairs of legs for both males and females. E. Female Tarsus. No EYFP expression was observed in any tarsus in female legs. F. Front Male Tarsus. EYFP expression was observed only in the front tarsus in males.

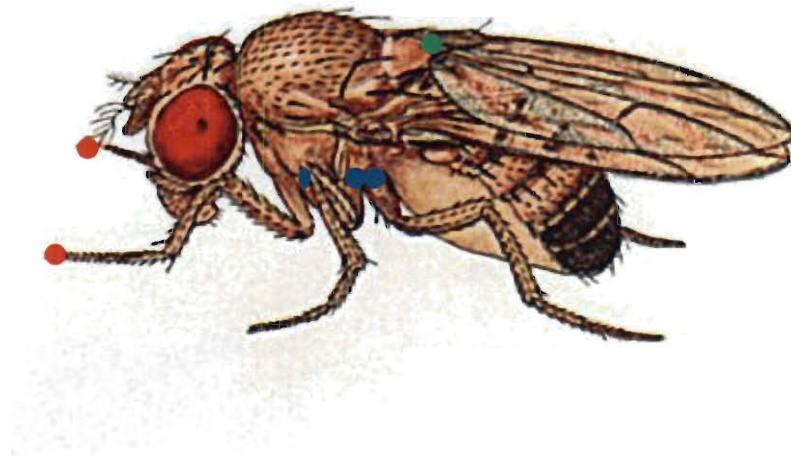


Figure 2-2: Diagram to show the sites of 91Y-induced GAL4 expression in the adult fly. When driven by the 91Y P{GawB} element, EYFP expression is observed in the stretch receptor of the wing (green dot), the leg chordotonal organ (blue dots), and front male tarsus (red dots). (Adapted from [http://insects.eugenes.org/species/about/species-gallery/Drosophila\\_melanogaster/](http://insects.eugenes.org/species/about/species-gallery/Drosophila_melanogaster/))



Location		Males (n=11)	Female (n=10)
Right foreleg:	Tarsus	9	0
	CHO	11	9
Left Foreleg:	Tarsus	10	0
	CHO	10	9
Right Middle Leg:	Tarsus	0	0
	CHO	10	9
Left Middle Leg:	Tarsus	0	0
	CHO	9	9
Right Hindleg:	Tarsus	0	0
	CHO	11	10
Left Hindleg:	Tarsus	0	0
	CHO	11	10

Table 2-1: EYFP is expressed in legs under the 91Y P{GawB} insertion. Eleven complete leg sets were examined in males and 10 complete leg sets were examined in females. For both males and females, expression in the leg chorodnotal organ (CHO) was observed in all individuals examined. Expression in the tarsus was limited to the front legs of males only. Of 11 sets of legs examined for males and 10 sets of legs examined for females, 19 out of 21 male front legs had EYFP expression. In contrast, none of the male middle or hind legs and none of the female legs had EYFP expression.

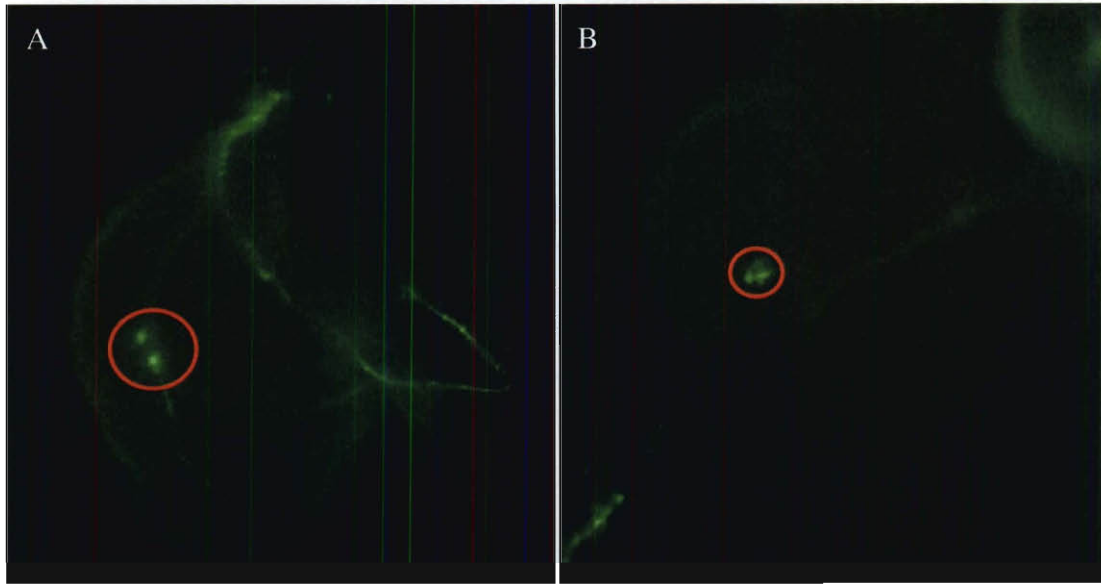


Figure 2-3: 91Y driven EYFP expression in larvae. A&B. EYFP expression was observed in a small number of cells in the leg disc of third instar larvae. It is believed that these are the precursor neurons for the adult leg CHO.

expression in the stretch receptor in the wing and the chordotonal organ of the leg suggest that *lov* functions in helping the fly determine the location of its appendages relative to its body. Additionally, the expression in the leg chordotonal organ suggests a route by which the 91Y mutation may affect gravitaxis. Further, the expression of EYFP early in the development of the leg chordotonal organ could indicate that *lov* has a determinative role in its development.

Of most interest, however, is the EYFP expression in the tarsus, which was only observed in the front legs of males. This suggests that there may be a male specific promoter for *lov* as is true for *fruitless*. There are male- specific taste receptors in the front legs of males that allow them to identify females as proper courtship objects. Therefore the male-specific EYFP expression only in the tarsus of the foreleg suggests that *lov* is expressed in these neurons and *lov* may be important in the tasting aspect of courtship.

### CHAPTER 3: GENERATION AND MOLECULAR CHARACTERIZATION OF NEW *lov* MUTANTS

The gravitaxis screen that identified the 91Y mutation was designed to isolate mutations that only affect gravity response. However, it is likely that *lov* has a role in other processes. Stronger mutations in *lov* were needed to better understand its biological roles in *Drosophila*. Two methods of mutagenesis, transposon excision and gamma ray mutagenesis, were used to generate new deletion alleles of this gene.

In transposon excision, a transposon, 91Y, is mobilized by introducing a transposase into the genome to mobilize the insertion element. This mobilization causes the element to leave its insertion site, often disrupting adjacent DNA. Given that the transposon contains the  $w^+$  gene, the mobilization event also changes eye color from red to white, allowing for a quick screen for potential mutants. The drawback to this method is that it often produces only small deletions in the gene of interest. The crossing scheme that was used for transposon excision of the 91Y insertion is shown in Figure 3-1.

To generate much larger deletions in *lov*, gamma ray irradiation was used. Gamma rays cause double-strand breaks in DNA, which may give rise to large deletions during DNA repair. 91Y males were exposed to gamma rays and after crossing to a balancer line, their progeny were screened for a change in eye color from red to white (Figure 3-2). A change in eye color again indicates that the insertion element has been removed, possibly along with some of the genomic DNA surrounding the insertion site. The advantages of gamma ray mediated excision over transposon excision are the potential to create larger deletions and the smaller number of crosses required to generate stocks. The end result was roughly 200 new excision lines through transposon mediated excision and 30 lines through gamma ray mediated excision, most of which are lethal (Table 3-1).

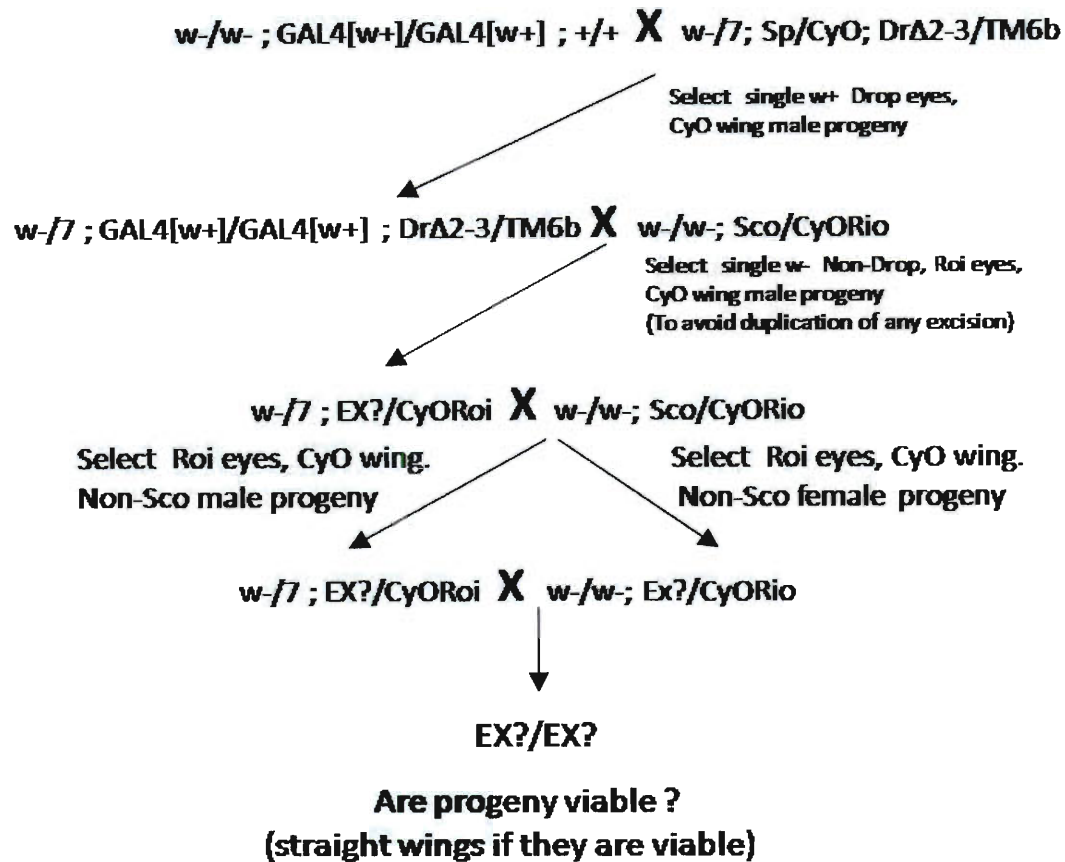


Figure3-1: The transposon excision scheme used to generate new alleles of *lov*. The  $\Delta 2-3$  transposase is introduced in the first cross and then individuals lacking the transposase (i.e. non-Drop) and the transposon (i.e.  $w^-$ ) are selected for analysis.

**Males exposed to gamma rays:**

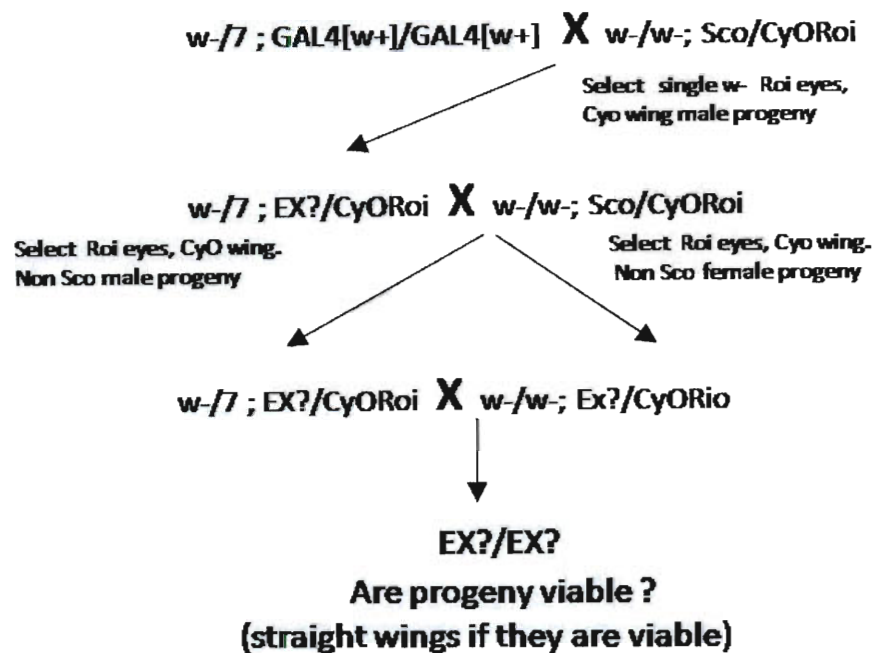


Figure3-2: The genetic scheme used to generate new *lov* alleles by gamma ray irradiation. 91Y males are first exposed to gamma rays and then crossed to a balancer line. Their progeny are screened for a change in eye color from red to white, which indicates that the insertion element has been removed, possibly along with some of the genomic DNA surrounding the insertion site.

Table 3-1: Summary of Mutants Generated by Both Transposase-Mediated Mutagenesis and Gamma Ray Mediated Mutagenesis.

Genomic Structure	Number of Lines
P-element remaining	67
Precise Excisions	26
Confirmed Deletions	5
Sterile Lines	19
Lethal Lines	45

Note: The lethality in all of the lethal lines was found to be due to mutations outside the *lov* gene by detailed analysis. See Figure 3-3.

A series of PCR reactions was performed to determine if the mutants generated from the excision crosses are precise excisions, in which just the P-element is removed and the gene remains intact, or imprecise excisions, in which all or part of the P-element is removed and/or part of the genomic DNA is removed. To determine this, an initial PCR reaction was performed using a pair of primers, one immediately upstream of the insertion site and one ~500 base pairs downstream of the insertion site. If a line is a precise excision, the PCR reaction product will be approximately 0.8 kbp in size. For any such lines, this product was sequenced to confirm that the P-element had been completely removed. If a line did not generate a product with the above primers but the proper control did, deletion of DNA in the region was indicated. Further PCR reactions were therefore performed to examine the structure of the DNA at the insertion site. Primers in both the genomic and transposon DNA were designed to help characterize these lines. Sequencing was used to confirm the results of the PCR reactions. Lines which appear to have significant deletions were chosen for further analysis. Given that most of the gamma irradiation mutants were lethal, they are likely to be large deletions. Therefore, PCR reactions could not be used as part of their initial characterization.

Forty-five potential lethal lines were generated from the transposon excision and gamma ray mutagenesis. To confirm that the lethality was in fact due to a mutation at the *lov* locus, rather than a second mutation outside the *lov* locus, the lethal lines were crossed to a *lov* deficiency line (Bloomington Stock #4961) in which the region around the *lov* locus has been removed (Pauli et al., 1995, Duffy et al., 1996, Preiss et al., 1985, Cote et al., 1987, Ludwig et al., 1991, Gotwals and Fristrom, 1991, Germeraad et al., 1992) (Figure 3-3). If the lethality is due to a mutation at the *lov* locus, then  $lov^{leth?}/lov^{def}$



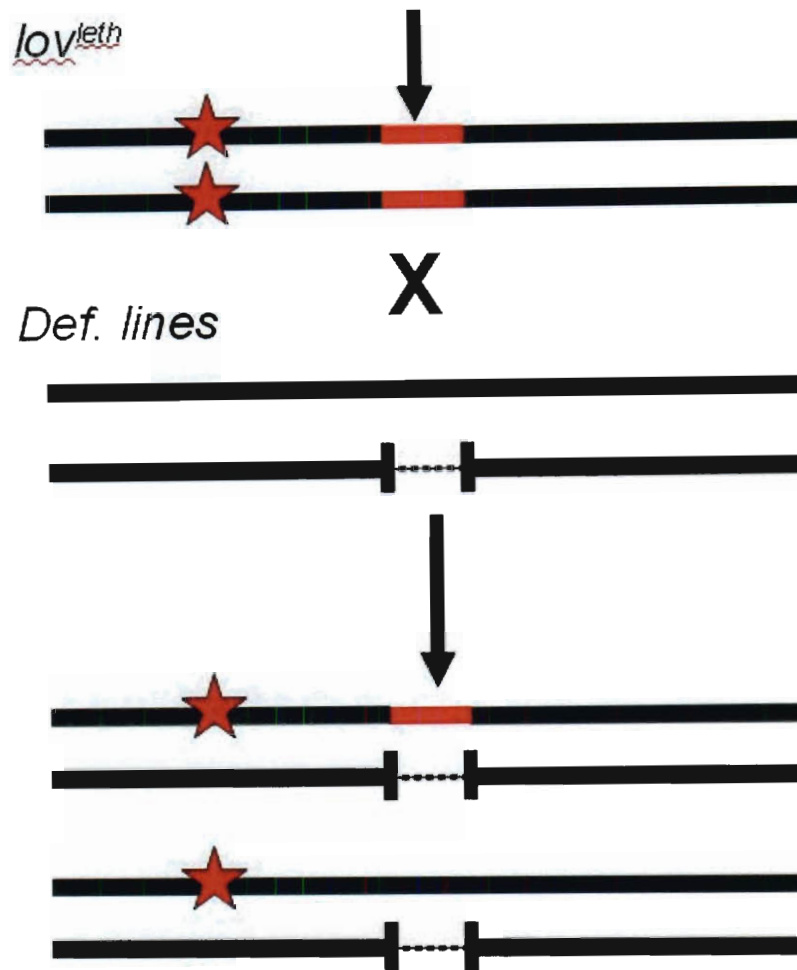


Figure 3-3: Determining if lethality is due to a mutation in *lov*. To determine if the observed lethality is due to a mutation at the locus or to a mutation outside the *lov* locus, each of the *lov* lethal lines was crossed to the *lov* deficiency line, which lacks the entire chromosomal region around *lov*. Offspring are hemizygous for each *lov* mutant can be generated and the mutant alleles of *lov* are analyzed. In these progeny, any unrelated mutation on the *lov* chromosome would be compensated by the wild type allele on the deficiency chromosome. If the lethality is due to a mutation at the *lov* locus, then *lov<sup>leth?</sup>/lov<sup>def</sup>* flies will die. If the lethality is due to a mutation outside the *lov* locus, then *lov<sup>leth?</sup>/lov<sup>def</sup>* flies will survive.

flies will die. If the lethality is due to a mutation outside the *lov* locus, then  $lov^{leth?}/lov^{def}$  flies will survive. For all 45 lines, the  $lov^{leth?}/lov^{def}$  progeny survived to adulthood, indicating that the lethal mutation is not at the *lov* locus.

In addition, 19 sterile lines were generated from the two rounds of mutagenesis. For all of these lines, homozygous adults were found. However, when these homozygous adults were mated to one another, they did not generate offspring. In most of these lines, a portion of the P-element remains at the 91Y insertion site. One of these lines,  $lov^{66}$  is a 387 base pair deletion, which has been studied in detail (see below).

In all, five new mutations that delete genomic DNA from the *lov* locus were identified in the mutagenesis screens.  $lov^{38}$  has 2.5 kb of the 5' end of the P-element remaining and a 0.66 kb deletion upstream of the insertion site.  $lov^{47}$  appears to have 6.5 kb of the 5' end of P-element remaining and a 1.4 kb deletion upstream of the insertion site. Both mutations produce viable adults, with no obvious developmental defects.  $lov^{66}$  is a 387 base pair deletion that begins at the insertion site and ends 76 base pairs upstream of RB/RD transcription start site (Figure 3-4).  $lov^{66}$  shows a fertility defect. These three lines were chosen for further analysis to help understand the function of *lov* in *Drosophila*.  $lov^{10}$  and  $lov^{65}$ , which were initially believed to be precise excisions, were both found to have a 16 base pair deletion 77 base pairs downstream of the insertion site and several small insertions and/or deletions upstream of the insertion site.

The original *lov* mutant, 91Y, identified a role for *lov* in gravitaxis, indicating a role for *lov* in behavior. When stronger mutations in *lov*, particularly  $lov^{66}$ , were generated, *lov* was also revealed to play a role in fertility, either by affecting gamete development or mating behavior or both. Therefore, the subject of this thesis is to begin

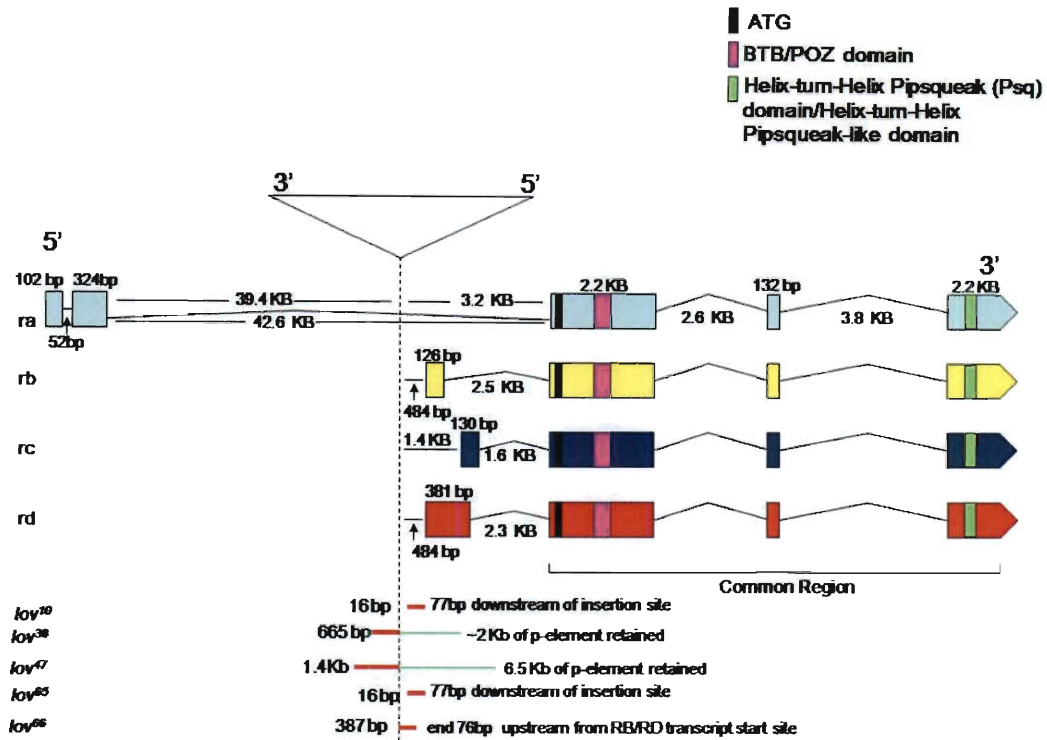


Figure 3-4: New lov mutants generated in these studies. lov<sup>38</sup> has 2.5 kb of the 5' end of the P-element remaining and a 665 base pair deletion upstream of the insertion site. lov<sup>47</sup> has a 6.5 kb of the 5' end of P-element remaining and a 1.4 kb deletion upstream of the insertion site. lov<sup>66</sup> is a 387 base pair deletion that begins at the insertion site and ends 76 base pairs upstream of RB/RD transcription start site. Both lov<sup>10</sup> and lov<sup>65</sup>, which were once believed to be precise excision were found to have a 16 base pair deletion 77 base pairs downstream of the insertion site and several small insertions and/or deletions upstream of the insertion site.

to understand lov's role in fertility. Each of the five lov mutants, along with 91Y will be analyzed behaviorally to determine how the various deletions affect courtship. lov<sup>66</sup> will also be examined to determine how its deletion affects developmental aspects of fertility, such as spermatogenesis and oogenesis.

## CHAPTER 4: INVESTIGATION OF THE LOV EXPRESSION PATTERN IN THE EMBRYONIC NERVOUS SYSTEM.

### Generation of a Lov antibody

Previous work has suggested a role for lov in behavior and fertility (Chapters 1 and 3). To better understand the function of Lov, an antibody was needed. Examining the Lov protein expression pattern using an antibody in wild type and mutant flies will give an idea of when and where the protein is found in wild type flies and whether the mutations disrupt this distribution. It will also identify the subcellular location of the protein. Knowing the subcellular location of the protein will provide insight into the roles of lov.

Based on sequence data, lov is predicted to be a transcription factor due to the presence of the Helix-turn-Helix Pipsqueak (Psq) and Helix-turn-Helix Pipsqueak-like domains. Therefore, it is likely that Lov expression will be found in the nucleus. Other data demonstrates that Lov expression is neural. In 91Y, GAL4 expression is found in the brain (Flytrap.org), in stretch receptors in the wing and legs, and possibly in taste receptors in the forelegs of males (Chapter 3). Finally, using RNA in situ analysis, the Crews lab has found that lov mRNA is expressed in a specific pattern in the ventral unpaired median (VUM) neurons (a type of motor neuron) in the embryonic CNS midline cells and later, is expressed throughout the embryonic CNS (Kerney et al. 2004). However, the complete lov expression pattern is unknown. By creating an antibody against Lov, the complete expression pattern can be established and insights into its functions achieved.

### Designing of antigenic region for a Lov antibody

To generate an antibody against the Lov protein, a small antigenic fragment (218 amino acids) was identified. The protein coding sequence of lov was compared to the protein sequences of all other predicted proteins in the *Drosophila* genome to determine which regions of the Lov have high sequence homology with other proteins. The non-matching regions were then checked to see if they lie across intron/exon boundaries. Only those regions which are contained within an exon were used, because they are more likely to be part of a single functional domain. The protein coding region chosen is located in the first exon common to all four transcripts. It is located 948 base pairs downstream from the ATG start site and 270 base pairs downstream of the predicted BTB domain (Figure 4-1).

Primers were designed based on the selected region and the appropriate restriction sites were added to the beginning of the primers to allow insertion into an expression vector (Figure 4-2). The antigenic fragment was designed to be cloned between BamH I and EcoR I restriction sites. The pGEX-6P-1 expression vector (GE Life Sciences) was used because it adds a Glutathione-S-transferase (GST) tag to the protein, allowing the Lov fusion protein to be quickly purified on a Glutathione-Sepharose column. It also contains a proteolytic cleavage site which allows for the protein to be released from the tag using a proprietary protease, PreScission Protease. This protease also contains a GST tag which allows for simultaneous removal of the protease and the GST portion of the fusion protein. This vector has also been used previously in our lab and has yielded good results in the past.

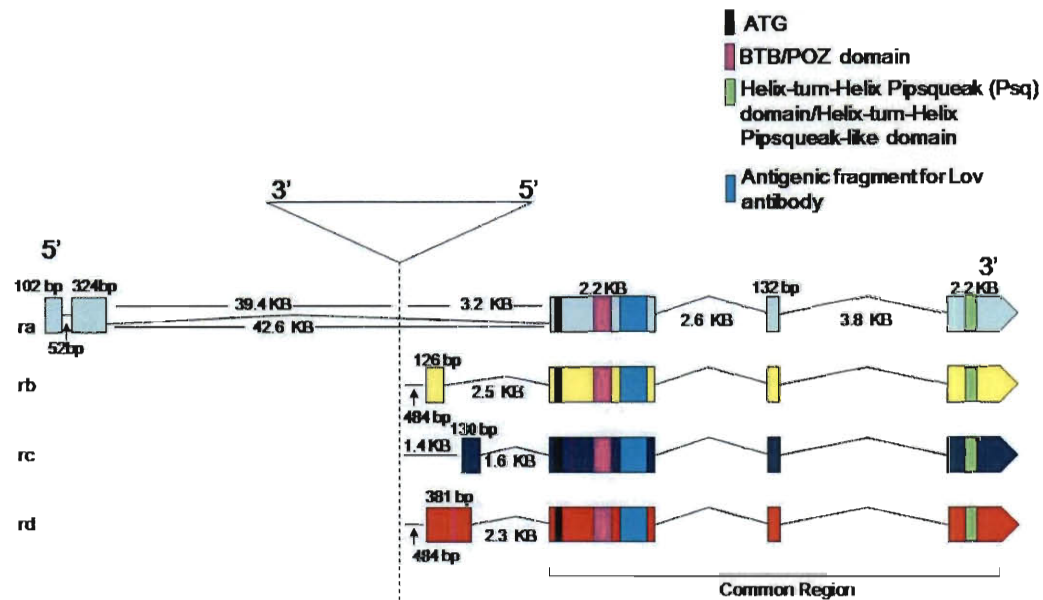


Figure 4-1: Location of the Lov protein fragment chosen as an antigen for antibody production. The location of the antigenic fragment is indicated by the bright blue square. The fragment is located in the first exon common to all lov transcripts. It also contains the ATG start site (black square) and the BTB domain (purple square). It is located 948 base pairs downstream from the ATG start site and 270 base pairs downstream of the predicted BTB domain. All four transcripts encode the same protein; therefore antigenic fragments unique for each transcript could not be designed.





### Cloning and purification of Lov protein fragment

Using standard cloning techniques, the PCR product containing the antigenic fragment was inserted into the pCR4-TOPO vector (Invitrogen). The antigenic fragment was then transferred to the pGEX-6P-1 expression vector to create pGEX-6P-1-lov and sequenced. Upon sequencing, there was a single amino acid change (V32A) but that change is unlikely to have an effect on the overall antigenic properties of the antibody. The construct was transformed into BL21(DE3) competent cells and expression of the GST-Lov fusion protein was induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) (Figure 4-3). Lysates containing the fusion protein were then used to generate the purified Lov protein fragment as described in Material and Methods (Figure 4-4). The purified Lov fragment was then sent to CoCalico Labs for antibody production in guinea pig and rabbit.

Based on the peptide sequence, the Lov portion of the fusion protein is predicted to have a molecular weight of ~24 kDa. The GST portion is known to have a molecular weight of 27 kDa. Therefore, the combined molecular weight of the fusion protein should be about 50 kDa. However, as shown Figures 4-3 and 4-4, both the fusion protein and the Lov fragment are found at higher molecular weights. The fusion protein appears to have a molecular weight of 70 kDa and the Lov fragment appears to have a molecular weight of 55 kDa. Prior to use, sequencing had established that the construct was as expected. The expression vector has only one copy of the PCR product containing the antigenic fragment and there only a minor change to the sequence, that would not have an effect on the final antibody. When the amino acid sequence was examined by a coiled-coil predictor program ([www.ch.embnet.org/software/COILS\\_form.html](http://www.ch.embnet.org/software/COILS_form.html); Lupas

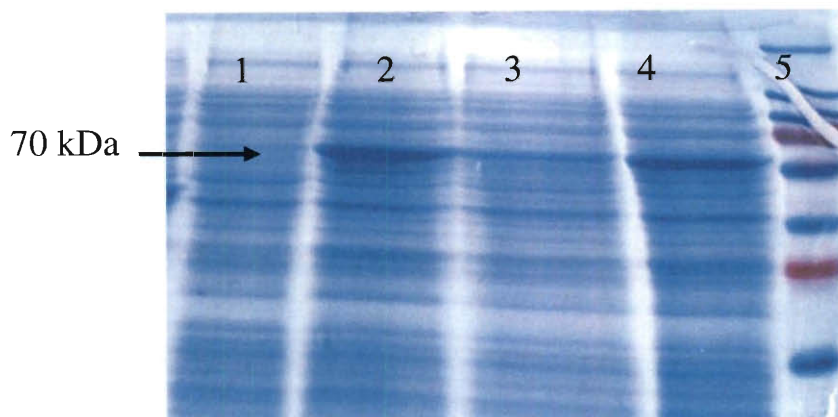


Figure 4-3: Induced expression of GST-Lov fusion protein. Uninduced and Induced bacterial lysates containing the Lov expression were run on 12.5% SDS acrylamide gel. Lanes 1 and 3 are un-induced lysates of bacterial cells carrying the Lov expression vector. Lanes 2 and 4 are induced Lov samples. In the induced samples, there is a large band at 70 kDa, corresponding to the GST-Lov fusion protein.

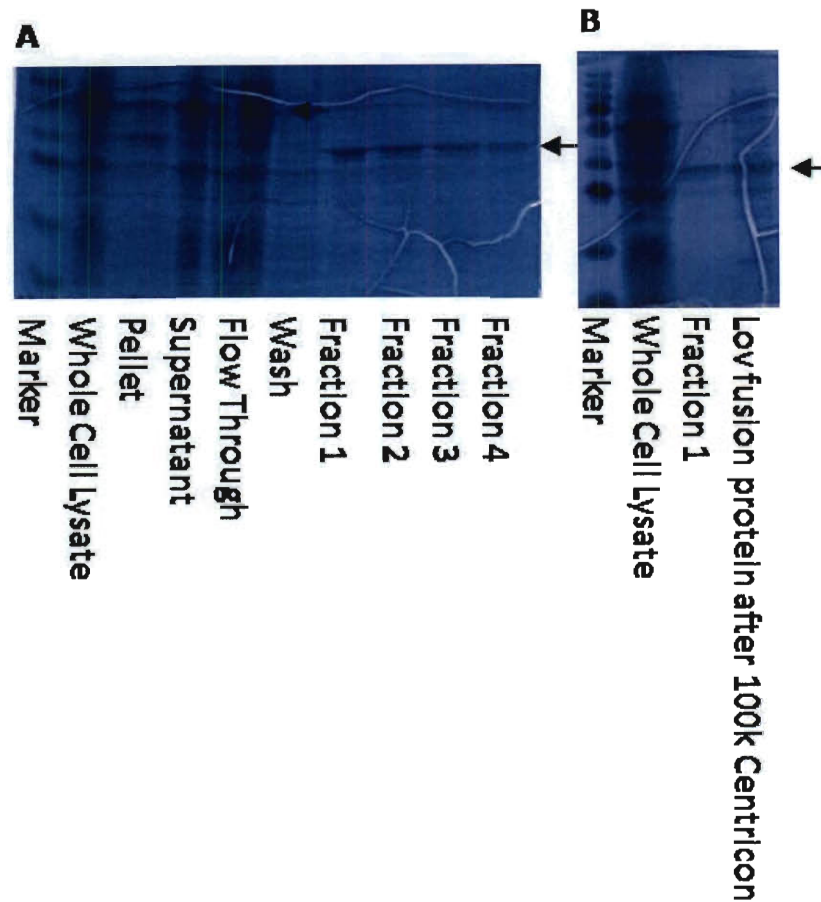


Figure 4-4: Purification of the bacterially expressed Lov protein fragment. A. Purification of the Lov fusion protein using a glutathione column. In the whole cell lysate, the supernatant, and, to a lesser extent, the flowthrough, there is a strong band at 70 kDa (higher black arrow) which is the Lov fusion protein. The fusion protein is not present in the pellet, indicating that the fusion protein is soluble. Likewise, in the wash lane, the fusion protein is not present, an indication that the fusion protein bound well to the column. The last four lanes are the first four fractions to be eluted off the column after protease treatment. The Lov fragment is found at 55 kDa (lower black arrow) after removal of the GST tag. B. Concentration of Lov peptide fragment protein with 100 kDa Centricon column. After running through a 100 kDa Centricon column, the untagged Lov fragment is more concentrated than in Fraction 1.

et al., 1991), two regions were found that are predicted to form helices. Therefore, it is possible that the Lov fragment is forming dimers, accounting for the higher molecular weight observed in the gels.

#### Testing of Lov Antibodies

The quality of the antibodies produced by each animal was tested using immunoblots, using the purified Lov fragment at various concentrations as well as extracts of whole flies, to determine the affinity of each antiserum for native *Drosophila* Lov. First, the pre-immunization bleeds were tested to ensure that the animals used did not have any antibodies that would cross react with Lov. As can be seen from Figure 4-5, neither prebleed appeared to cross react with the Lov fragment.

Next, as the various bleeds came in, the quality of the antibodies was tested by further immunoblotting. The purified Lov fragment at various concentrations was probed, as well as extracts of whole flies to see if the antibodies detect Lov protein in fly tissue. When the various bleeds were tested against the purified Lov fragment, a single band appeared at the size of the Lov peptide (55 kDa). Only at a high protein concentration (1 µg) did multiple bands appear, suggesting that the antibodies being generated were specific for Lov and that there was a low level of background cross-reactivity (Figure 4-5).

The final bleeds were tested on extracts of solubilized *Drosophila* heads and testes. These two tissue types were chosen because they are predicted to have the highest expression levels of lov (Flyatlas.org). When tested on wild type heads and testes, two major bands appear around 70 kDa (Figure 4-6). This is smaller than the expected size of 110 kDa based on sequence data. Additionally, there is a large amount of background,

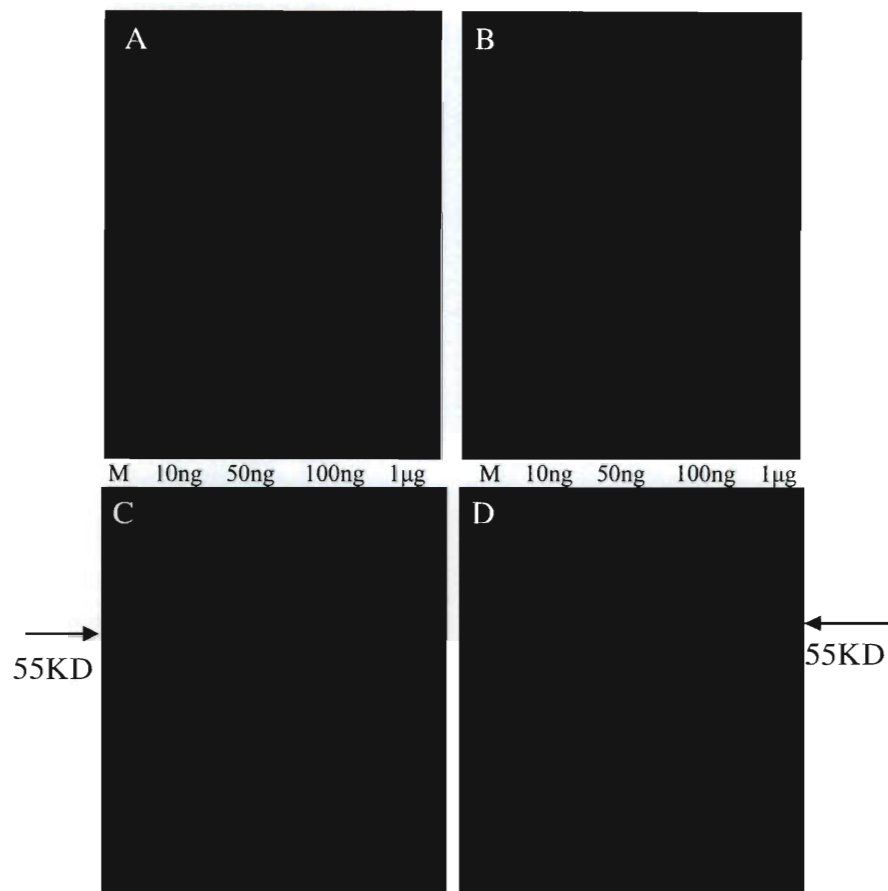


Figure 4-5: Immunoblot testing of the guinea pig and rabbit anti-sera. (A,B) Testing of pre-immunization sera. The preimmune sera from the guinea pig (A) and rabbit (B) used to raise Lov antibodies do not cross-react with the Lov fragment. (C,D) Testing of the first bleeds. For Guinea  $\alpha$  Lov (C) and Rabbit  $\alpha$  Lov (D), for the lower concentrations of the Lov fragment, a single band appeared at 55 kDa, which is the size of the fragment. Only with a higher concentration of 1  $\mu$ g did multiple bands form.

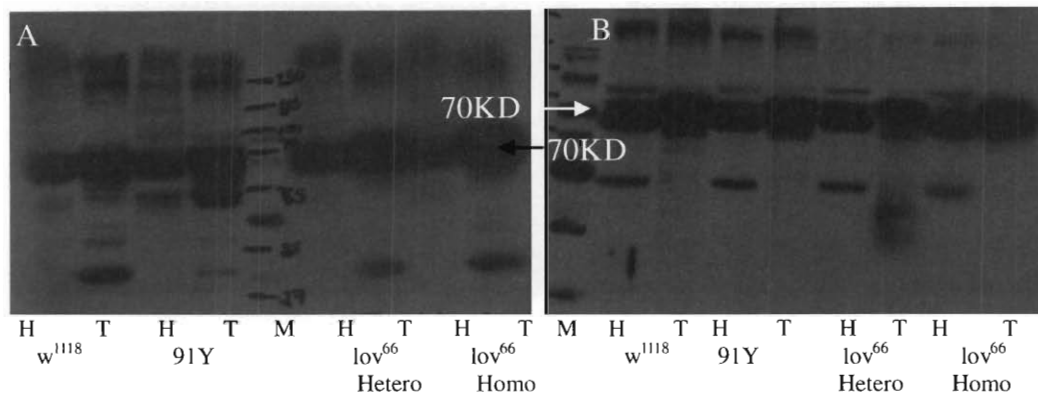


Figure 4-6: Protein bands recognized by the unpurified final bleed anti-sera from guinea pig (A) and rabbit (B) in tissues of wild type and mutant flies. The expected size of Lov is 110 kDa. However, there are two major bands which appear to be around 70 kDa. The larger of the two bands in *lov<sup>66</sup>* homozygous heads is not detected by the rabbit  $\alpha$  Lov. Similarly the smaller of the two bands in the *lov<sup>66</sup>* homozygous testes is not detected by the rabbit  $\alpha$  Lov and is detected at a reduced level in the *lov<sup>66</sup>* heterozygous testes when probed with rabbit  $\alpha$  Lov. (H=heads; T=Testes)

suggesting that these unpurified Lov antisera are cross-reacting with other proteins in the tissues. Interestingly, the larger of the two bands is not detected in the lov<sup>66</sup> homozygous heads using the rabbit antibody, and the smaller of the two bands is not detected in lov<sup>66</sup> homozygous testes and detected at a reduced level in the lov<sup>66</sup> heterozygous testes when probed with the rabbit  $\alpha$  Lov. The bands detected by the Lov antibody may represent degradation products or post translation modifications that are differentially produced in different tissues.

#### Affinity Purification of Lov antibody

As shown above, initial immunoblots showed that the antibodies picked up two major bands around 70 kDa. This is smaller than the predicted 110 kDa, raising questions as to whether or not the antibodies are recognizing Lov. Therefore, to help clarify this issue, affinity purification of the Lov antibodies from both guinea pig and rabbit was used (see Material and Methods) to remove any background and help identify which bands represent Lov products.

The affinity purified antibodies from both guinea pig and rabbit are more specific than the original antibody (Figure 4-7, results for guinea pig antibody not shown). For both antibodies, three bands are seen: one at 70 kDa, one at 55 kDa and one at 35 kDa. Interestingly, the smaller of the two 70 kDa bands previously detected with the unpurified rabbit antibody is not detected by the purified rabbit antibody. Although these bands are smaller than the predicted 110 kDa, these bands may represent Lov products. They could reflect degradation or post-translational modification of Lov. Unlike the original antibody, the purified antibodies do not show a difference between wild-type and mutant

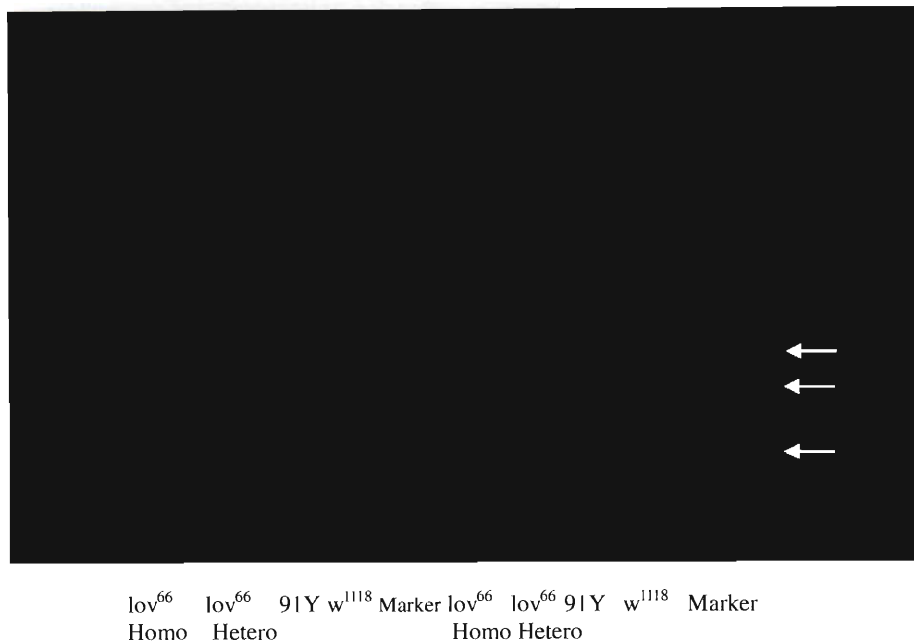


Figure 4-7: Comparison of the unpurified and purified Lov rabbit antibody by immunoblotting. The original antibody (left) and the affinity purified antibody (right) were compared by immunoblotting solubilized *Drosophila* head proteins from wild type and mutant flies. 91Y is the original mutant identified in the gravity screen. lov<sup>66</sup> is a 400 base pair deletion mutant that has defects in courtship and gamete development. As can be seen, the affinity purified antibody is much more specific. Three bands are detected in the affinity purified antibody, one at 70 kDa (very faint in this blot) one at 55 kDa and one at 35 kDa. The 70 kDa band is the larger of the two 70 kDa bands previously detected with the unpurified rabbit antibody.



tissues. However, given that the bands that cross react with that antibody are smaller than predicted and little is known about Lov the expression pattern, this difference says nothing about the specificity of the Lov antibodies. After affinity purification, the Lov antibodies from both species detected identical bands in immunoblotting, but the guinea pig  $\alpha$  Lov was more concentrated and was therefore used for all further experiments.

Embryo staining reveals that the Lov antibody is specific

Although affinity purification improved the quality of the Lov antibody, the bands that cross react with that antibody are smaller than predicted. As mentioned above, they may represent degradation or post-translational modification of Lov. However, the results of the immunoblots describe above were inconclusive. To further investigate the specificity of the antibody, a different method, embryo staining, was used.

Using in situ hybridizations, the Crews' lab determined that lov mRNA is expressed in specific pattern in the ventral unpaired median (VUM) neurons (a type of motor neuron) in the CNS midline cells in mid-embryogenesis and throughout the ventral nerve cord later in embryonic development (Kerney et al. 2004) (Figure 4-8A; Steve Crews, personal communication). Therefore, to test the specificity of the antibody, staged embryos were stained with the antibody to see if this known expression pattern in the CNS is present. If this same expression pattern is detected, this would indicate that the antibody is detecting Lov.

A 0 to 24 hr embryo collection was immunostained with the Lov antibody (see Material and Methods) and the Lov expression patterns was examined. A late CNS expression pattern identical to that seen by the Crews lab was detected, indicating that the antibody is specific for Lov (Figure 4-8B and C). Further, the protein proved to be

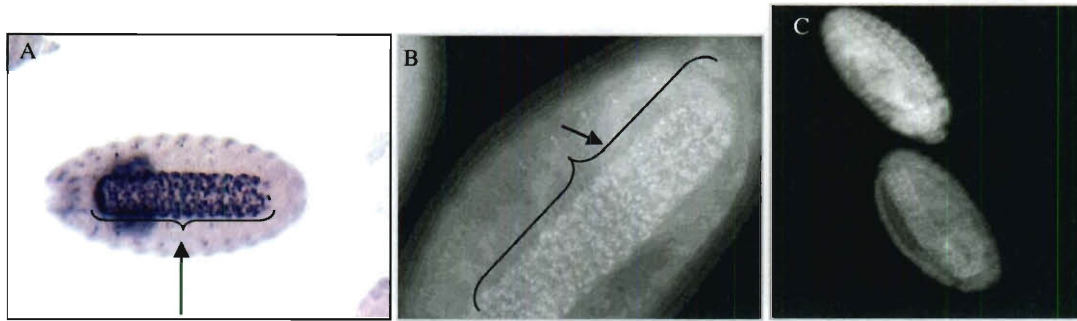


Figure 4-8: Comparison of the expression pattern of *lov* mRNA with that of the Lov antibody in late embryogenesis. A. *lov* mRNA expression pattern in late stage embryos showing *lov* expresses in the CNS(indicated by the arrows) (Steve Crews, personal communication). B&C. Lov expression pattern as detected with the antibodies, the pattern is identical to that of the mRNA, indicating that the antibody is specific for Lov.

nuclear, suggesting that lov may have a role as a transcription factor, as predicted by sequence analysis.

To further investigate the specificity of the Lov antibody, the expression pattern of Lov was examined when lov was over-expressed using the UAS-GAL4 system (Chapter 8). A UAS-lov transgene was expressed using an elav-GAL4 line, which will result in lov expression in every neuron. As can be seen in Figure 4-9, there is a large increase in number of Lov staining neurons in the elav-GAL4/ UAS-lov embryos compared to wild type. This confirms that the antibody is specific for Lov.

#### Lov expression in the Embryonic PNS

In the process of determining whether the Lov antibody was specific, it was discovered that Lov is expressed in the PNS of the developing embryonic nervous system (Figures 4-8 and 4-10). As mentioned above, Steve Crews' lab, through in situ hybridizations, has shown that lov is expressed in CNS of developing embryos (Kerney et al. 2004) and Lov immunostaining demonstrated a similar pattern. The Crews lab has not characterized the lov expression in the PNS and therefore that analysis was undertaken as part of this thesis study. The neurons of the PNS are well characterized and thus examining Lov expression in the PNS should provide insight into the function of lov.

The *Drosophila* embryonic PNS contains three main types of sensory neurons: External Sensory Organs (ES), Chordotonal Organs (CH), and Multiple Dendritic cells (MD) (Jan and Jan, 1993; <http://www.normalesup.org/~vorgogoz/FlyPNS/page1.html>). The ES form the external sensory organs, bristles, which detect mechanical and chemical signals. The CH are stretch receptors or auditory sensors (Sun, Jan, and Jan, 2000). The function of the MD cells is currently not well known but they could be thermoreceptors,

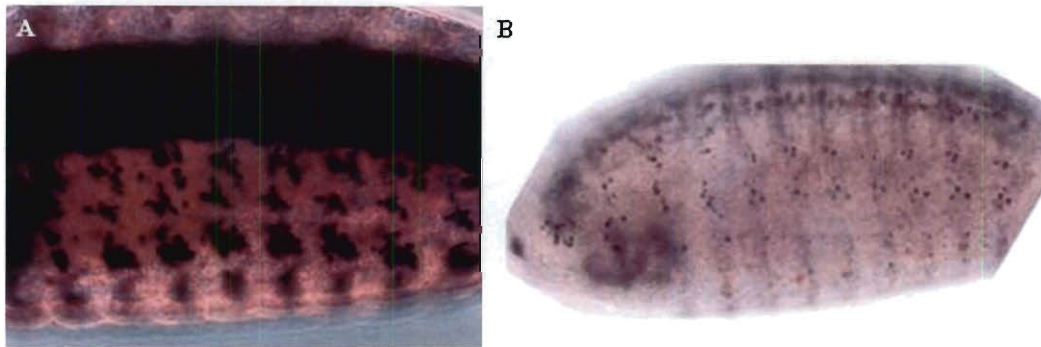


Figure 4-9: An increase in Lov-expressing neurons in *elav-GAL4; UAS-lov* compared to wild type embryos. Late stage embryos are shown, ventral side upper right, anterior left. When a *UAS-lov* transgene was ectopically expressed using an *elav-GAL4* line, resulting in *lov* expression in every neuron of the CNS and PNS, there is an increase of Lov staining (A) as compared to wild type (B). This confirms that the antibody is specific for Lov.

nociceptors, or osmoreceptors among other things (<http://www.normalesup.org/~vorgogoz/FlyPNS/page1.html>). Each of the different sensory neurons is generated under the regulation of a unique proneural gene. Proneural genes are transcriptional activators that contain a basic helix-loop-helix motif. (Villares and Cabrera, 1987; Alonso and Cabrera, 1988; Gonzalez et al., 1989; Sun et al., 2000). The proneural genes are expressed in proneural clusters in the undifferentiated ectoderm of the embryo to specify the formation of sense organ precursors (SOPs), which in turn gives rise to the sensory neurons (Jarman et al., 1993; Sun et al., 2000). The genes in the achaete-scute complex are the proneural genes required for ES formation (Campuzano and Modolell, 1992). Chordotonal Organ formation is driven by the proneural gene atonal (Jarman et al., 1993). Finally, the proneural gene amos is responsible for the formation of the Multiple Dendritic cells (Huang et al., 2000). One cell in each of these proneural clusters becomes the sensory neuron through an increase in expression in the appropriate proneural gene. This cell prevents neighboring cells from adopting the same fate through lateral inhibition executed by such genes as Notch and Delta (Lehman et al, 1983; Dietrich and Capos-Ortega, 1984; Simpson, 1990). The sensory neurons are further divided into specialized subsets of cells by neural identity genes.

#### Identification of Neurons Expressing Lov in the PNS

The Lov expression pattern in the PNS was examined and the cell types that express Lov were determined. Embryos were co-immunostained with the Lov antibody and the pan-neural marker, 22C10. The 22C10 antibody recognizes the protein encoded by the *Drosophila* gene *futsch* and is used to visualize neuronal morphology and axonal projections throughout the nervous system (Hummel et al., 2000). Because the PNS of

the embryo has been well described, the neurons in which Lov is expressed can be identified both on the shape of the neuron and its location when marked by the 22C10 antibody. The analysis of Lov expression in the embryonic PNS was focused on the neurons in the eight abdominal segments.

This analysis of doubly stained embryos established that Lov is expressed in subsets of all three major types of neurons in the embryonic PNS: the neurons of the External Sensory Organs (ES), and the Chordotonal Organs (CH), and Multiple Dendritic cells (MD) (Figure 4-10A and B). Most of the External Sensory Organs express Lov, whereas only two Chordotonal Organs and three Multiple Dendritic cells have Lov expression. It also appears that there is Lov expression in gustatory and olfactory neurons in the embryonic “head” (Figure 4-10-A).

As a confirmation that some of the Lov-containing cells were Chordotonal Organs, the expression in the PNS was further examined in an atonal null mutant. atonal embryos lack Chordotonal Organs; those elements of the PNS Lov expression pattern thought to be within Chordotonal neurons should be absent in this mutant. In the atonal embryos, Lov expression is absent at the positions normally occupied by the Chordotonal neurons. This finding confirms our determination that Lov is expressed in some of the Chordotonal Neurons (Figure 4-10C).

Within the lateral cluster of the sensory neurons of the embryo, Lov is expressed in only two of the five chordotonal organs. Interestingly, these two chordotonal organs are not developmentally related. 1c-3c (Figure 4-11I) are induced by atonal and the remaining two, 4c&5c, (Figure 4-11I) are induced by an EGF pathway (Okabe and Okano, 1997). Lov expresses in Chordotonal Organs 2c and 4c.

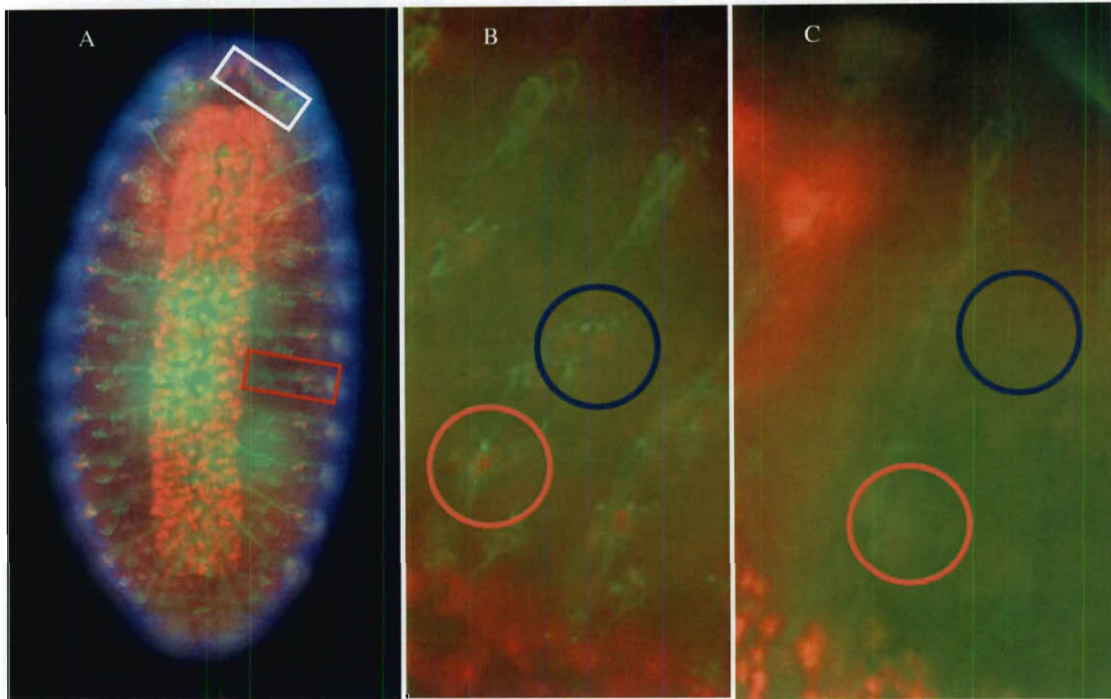


Figure 4-10: Lov antibody stains a subset of neurons within the embryonic PNS. A. Embryo stained with Lov antibody (orange), 22C10 antibody (green) and Hoechst (blue). Boxed in red is one hemi-segment of the PNS. Boxed in white are gustatory and olfactory neurons, which show Lov expression. B. High power image of the PNS of a wild-type embryo. The Chordotonal (CH) neurons detected by Lov are circled in blue and one set of External Sensory (ES) cells detected by Lov is circled in orange. C. Similar image of the PNS of an atonal mutant embryo. Note that the CH cells detected by Lov are not present in atonal mutants and that Lov no longer stains the regions containing these cells. However, the ES cells remain and Lov still stains that region.

Through careful analysis of the double immunostained embryos, all of the Lov expressing neurons of the abdominal segments of the fully developed embryo have been identified (Figure 4-11). Lov expression is found in all three types of sensory neurons in the PNS. The neurons in the PNS are arranged in three clusters- dorsal, lateral, and ventral. The ventral cluster is further subdivided into the v and v' clusters (<http://www.normalesup.org/~vorgogoz/FlyPNS/PNSorganization0.html>). Lov expression is found in all four clusters and can be described as expressing in five "rows" of cells containing a mixture of the different cells types (Figure 4-11I). Starting in the neurons most distal from the ventral nerve cord in the dorsal cluster (row 1), Lov is expressed in External Sensory neurons 11e, 12e, 13e (Figures 4-11 I), 14e and 15e (Figures 4-11I). In the lateral cluster (row 2), Lov is expressed in External Sensory neurons 8e, 9e, and 10e (Figures 4-11E (blue circle), F (black arrows), & I) and in Chordotonal cells 2c and 4c (Figures 4-11B (orange circle), D (black arrows), E (blue circle), and F (white arrow), &I). There are also several nuclei in this row that may be expressing Lov but are not obviously associated with a neuron. One is indicated in Figure 4-11F by a gray circle. In the v cluster (third row) of Lov expressing neurons, Lov expression is found in External Sensory cells 4e and 5e (Figures 4-11B (yellow circle), C (yellow arrow), E (purple circle), G (black arrows), and I). Expression is also found in the v' cluster (row 3) in two subclasses of Multiple Dendritic cells; 1t and 2t which are Tracheal Dendritic neurons and 7d which is a Dendritic Arborization neuron (Figure 4-11B (yellow circle), C (black allows), E (purple circle), G (purple arrows pointing to 1t and 2t), and I). There are also two Lov expressing nuclei in this row that are not obviously associated with a neuron. In the fourth row, which is part of the v cluster, Lov expression



is found in the external sensory neuron 1e (Figure 4-11I). Finally, in the v cluster (row 5) of cells, most proximal to the ventral nerve cord, Lov expression is found in two external sensory neurons: 2e and 3e (Figure 4-11E (orange circle), H (black arrows), and I).

#### Comparison of Lov expression in mutant and wild type embryos

It is possible that the various lov mutations affect the expression pattern of lov in the nervous system. As discussed earlier, lov is expressed widely in the CNS during embryonic development and in the adult stages. However, given that the individual neurons in the CNS have not been characterized at either stage, it is not possible to conduct meaningful analysis of the Lov expression pattern in the CNS at this point.

In contrast, the embryonic PNS is well-characterized and as discussed above, the Lov antibody allowed for the identification the Lov-expressing sensory neurons in the embryonic abdominal segments. As a first step to characterizing the effects of the lov mutants on neural expression, the pattern of Lov expression in the PNS of the abdominal segments was therefore determined for each of the mutants.

As discussed above, for wild type embryos, the individual Lov expressing PNS neurons in wild type were identified by co-immunolocalizations using the Lov antibody and antibody 22C10, which stains the outlines and axons of all neurons. However, for the mutant analysis, Lov staining alone was used, since it is easier to discern altered staining patterns in the absence of the counter stain. For this analysis, between 8-12 embryos for each of the various genotypes was compared to a similar number of wild type embryos of the same developmental stages.

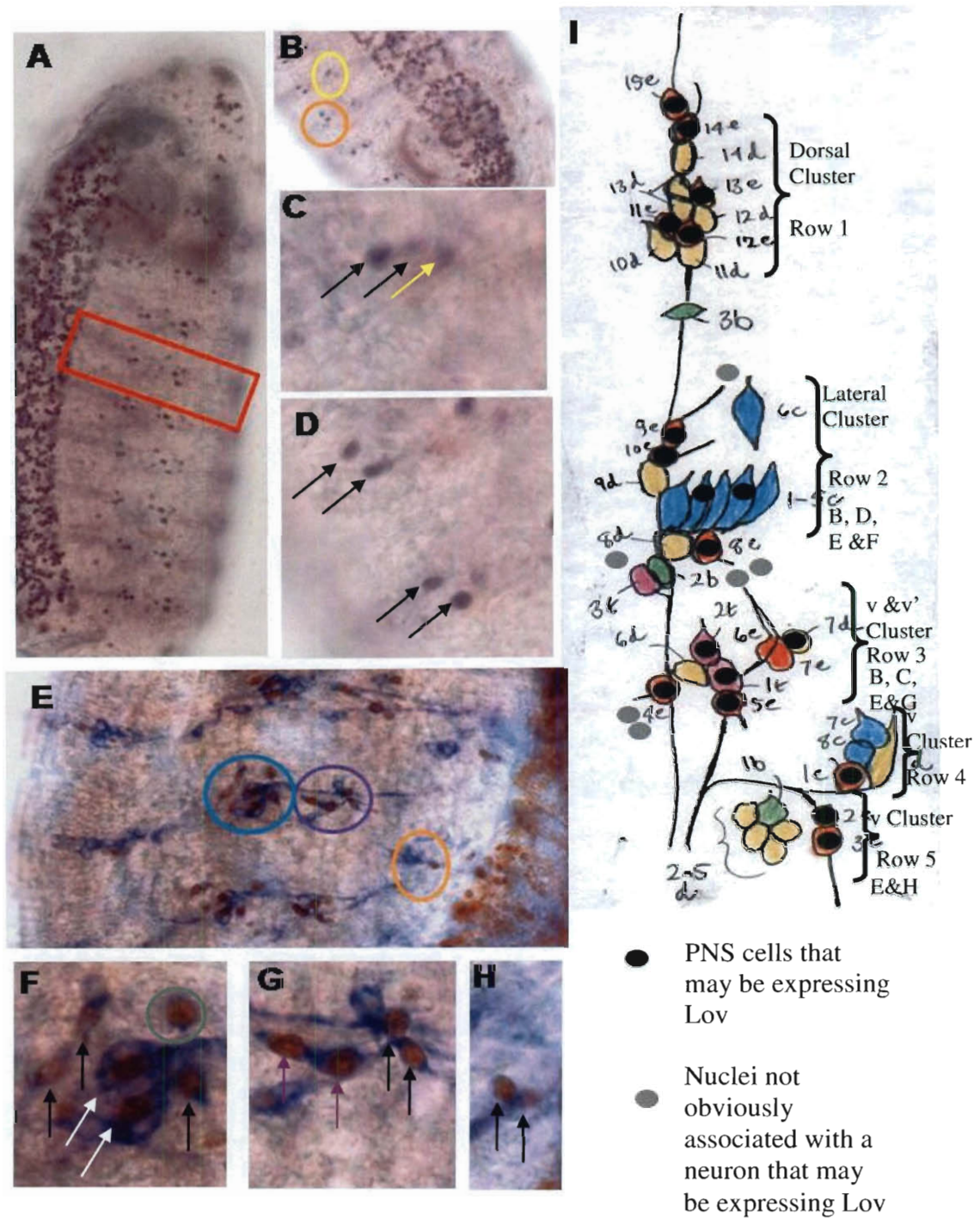


Figure 4-11: Identification of Lov expressing neurons by double immunostaining with Lov antibody and the 22C10 antibody.

Figure 4-11A. A late stage embryo stained with Lov antibody. Boxed in red is one hemi-segment of the PNS. B. An enlarged view of an embryo highlighting lateral, v and v' clusters (rows 2 & 3, orange and yellow circles). Lov is expressed in External Sensory neurons in both clusters and in the Chordotonal neurons in the lateral cluster. C. v and v' clusters with arrows indicating External Sensory neuron 5e (yellow arrow) and Multiple Dendritic Neurons 1t and 2t (black arrows) which are expressing Lov D. Lateral cluster with arrows indicating Chordotonal neurons 2c and 4c which are expressing Lov E. An enlarged view of an embryo highlighting row 2-5 of neurons in the embryonic PNS. Row 2 is circled in blue, row 3 is circled in purple, and row 5 is circled in orange. Row 4 is not visible in this embryo. F. In the second row of neurons, Lov expression is found in External Sensory neurons 8e, 9e, and 10e (black arrows) and in Chordotonal cells 2c and 4c (white arrows). There are also several nuclei in this row that may be expressing Lov that are not obviously associated with a neuron, one of which is indicated by a gray circle. G. In the third row of neurons, expression of Lov is found in two external sensory cells; 4e and 5e (black arrows) and in two subclasses of Multiple Dendritic cells, 1t and 2t (Tracheal Dendritic neurons) and 7d (Dendritic Arborization neuron) (purple arrows pointing to 1t and 2t). There are also two Lov expressing nuclei in this row that are not obviously associated with a neuron. H. In the row of neurons most proximal to the ventral nerve cord, Lov expression is found in two external sensory neurons: 2e and 3e (black arrows). I. Schematic Representation of a hemi-segment of the embryonic PNS and the cell that may be expressing Lov. Orange cells are the External Sensory neurons, the blue cells are Chordotonal cells and the yellow, green, and purple cells are the Multiple Dendritic cells.

In the late stages of embryogenesis (stage 16), as the ventral nerve cord begins to retract along the ventral surface of the embryo, most of the nuclei positive for Lov identified in Figure 4-11 can be seen in wild type embryos. By far the strongest staining nuclei are i) those of the two lateral (l) chordotonal neurons 2c and 4c (Figure 4-11E (blue circle) and F (white arrow)) in Row 2 and ii) two neurons of the ventral' (v') group, neurons 4e and 5e (Figure 4-11E (purple circle) and G (black arrow)) in Row 3. As the ventral nerve cord retracts further at late stage 16, staining is lost from the more weakly staining cells and these four nuclei are often the only four showing Lov staining in late embryogenesis (Figure 4-12 A'). In analyzing the mutant embryos, the focus was thus on these lateral and v' neuronal groups (circled in red in Figure 4-12 G). For the four mutations-  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{65}$ , and  $lov^{66}$  - no consistent differences between the staining patterns for these groups of neurons as compared to wild type were detected (Figure 4-12 B, B' C, C', D, D', E, and E'). In addition, the overall staining in the CNS of the ventral nerve cord was not detectably different from that of wild type, although no in-depth analysis was attempted. In contrast, marked differences in the Lov staining pattern were seen for  $lov^{47}$  (Figure 4-12F and F'). In the CNS, fewer cells were stained and the staining was much lighter in both the brain lobes and the ventral nerve cord. This effect was confirmed by comparing  $lov^{47}$  and wild type embryos stained in parallel under identical conditions. Most strikingly, essentially no staining was observed in the lateral group of neurons (Row 2 in Figure 4-12G) in  $lov^{47}$ , whereas the Lov expressing cells of the v' group (Row 3 in Figure 4-12G) were present. In particular the strong staining associated with the two chordotonal neurons, 2c and 4c, was absent in almost all abdominal segments analyzed. Further, ectopic expression of Lov could be detected in

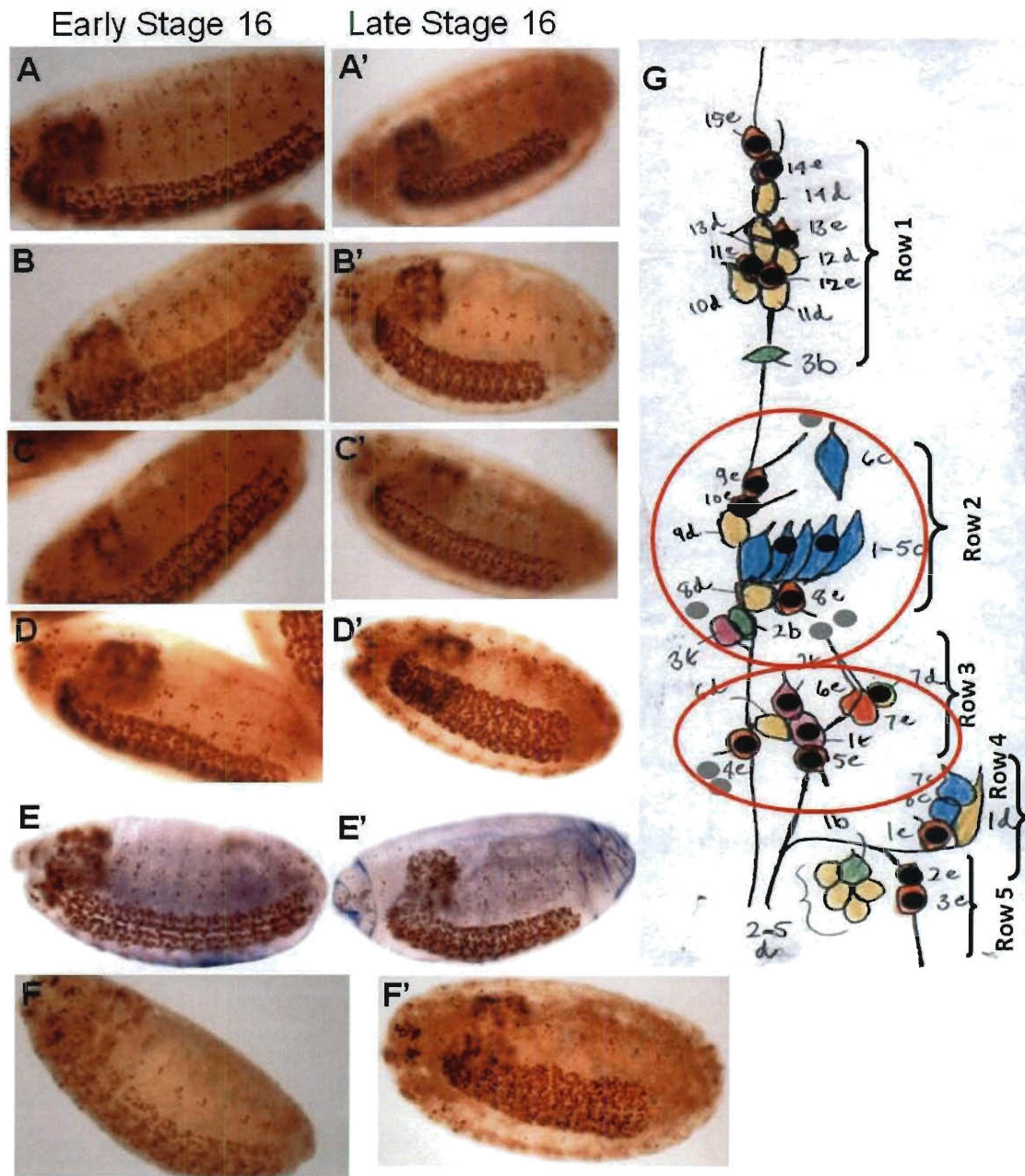


Figure 4-12: Comparison of *Lov* expression in the embryonic nervous systems of wild type and *lov* mutant embryos.



Figure 4-12: **A&A'**. An early and late stage 16 wild type embryo stained with Lov antibody **B&B'**. An early and late stage 16  $lov^{10}$  embryo **C&C'**. An early and late stage 16  $lov^{38}$  embryo. **D&D'**. An early and late stage 16  $lov^{65}$  embryo. **E&E'**. An early and late stage 16  $lov^{66}$  embryo (double stained with anti-GFP antibody to identify  $lov^{66}$  homozygous embryos). **F&F'**. An early and late stage 16  $lov^{47}$  embryo. G. Schematic Representation of a hemi-segment of the embryonic PNS and the cell that may be expressing Lov are indicated with a black dot. For four of the mutations-  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{65}$ , and  $lov^{66}$ - no consistent differences between the staining patterns for these groups of neurons as compared to wild type were detected. In contrast, no staining was observed in the lateral group of neurons (Row 2: 2c, 4c, 9e and 10e) in  $lov^{47}$ , whereas the Lov expressing cells of the v' group (Row 3: 4e, 5e, 1t, 2t, and 7d) were present.

this mutant. Two new lines of Lov staining nuclei within the interior of the embryo were seen in most of the embryos examined. It remains to be established what these cells represent – but they could be components of the gastrointestinal tract.

The Lov expressions patterns in the mutant embryos provide information on the function of the sequence deleted in the different mutant alleles. For every mutant except  $lov^{47}$ , there is no obvious change in Lov expression.  $lov^{47}$  deletes the largest region of the gene and is the only mutant that affects Lov expression in the embryonic nervous system. The deletion in  $lov^{47}$  starts at the 91Y insertion site and goes approximately 1.4 kb upstream (see Chapter 3 for a complete description of the deletion in each of the mutants).  $lov^{38}$  also has a deletion that starts at the 91Y insertion site and goes approximately 600 base pairs upstream. The difference in the Lov expression pattern in  $lov^{47}$  compared to  $lov^{38}$  suggests that the 800 base pair region of  $lov$  missing in  $lov^{47}$  but not missing in  $lov^{38}$  contains a regulatory element important for Lov expression in the embryo, particularly in the Chordotonal Organs.

What does immunostaining tell us about possible functions of  $lov$ ?

Based on immunostaining with the Lov antibody, Lov expression was found in the nervous system of the developing embryo, both in the CNS and the PNS. Its subcellular location was found to be in the nuclei, suggesting that it is a transcription factor. In the PNS, Lov expression was found to be expressed in subsets of all three sensory neuron classes. Immunostaining with the Lov antibody in embryos lacking *atonal* and thus lacking Chordotonal Organs, show a loss of the staining in the expected Chordotonal Organ locations. This result indicates several things. First, it confirms that Lov is expressed in a subset of Chordotonal neurons. Second, it indicates that  $lov$  is

neural identity gene, functioning in further specifying those Chordotonal neurons that express *Lov* into a subclass of Chordotonal neurons with a function different from the other Chordotonal neurons. Finally, the altered expression pattern observed in *lov*<sup>47</sup> suggests that the region removed in the *lov*<sup>47</sup> deletion contains a regulatory element that controls expression in the developing embryonic PNS.



## Materials and Methods

### Design of antigenic fragment

To generate a Lov antibody, a small antigenic fragment (218 amino acids) was identified by first comparing the protein coding sequence of lov to the protein sequences of all other predicted proteins in the *Drosophila* genome to determine which regions of the Lov have high sequence homology with other proteins. The non-matching regions were then checked to see if they lie across intron/exon boundaries and only those regions which are contained within an exon were used. Primers were designed based on the selected region and the appropriate restriction sites were added to the beginning of the primers to allow insertion into the pGEX-6P-1 expression vector. The upstream primer is 5'CTGGAAGTTCTGTTCCAGGGGCCCCTGGGATCC3' which adds a BamH I restriction site. The downstream primer is 5' GAATTCCCGGGTC GACTCGAGCGGCCGCAT3', which adds a EcoR I restriction site. Using standard cloning techniques, the PCR product containing the antigenic fragment was inserted into the pCR4-TOPO vector (Invitrogen). The antigenic fragment was then transferred to the pGEX-6P-1 expression vector to create pGEX-6P-1-lov and sequenced. Upon sequencing, there was a single amino acid change (V32A) but it is unlikely to have an effect on the overall antigenic properties of the antibody.

### GST-Lov fusion protein purification

Cells containing the GST-lov construct from an overnight LB+ampicillin culture were pelleted and resuspended in fresh M9+ampicillin and diluted 1:100 into 1-liter M9+ampicillin. Cultures were incubated at 37° C with shaking. Growth was monitored using periodic optical-density reading at 650 nm until cultures reached an absorbance of

0.5 to 0.8. When this absorbency was reached, expression of the Lov fusion protein was induced by adding isopropyl-beta-D-thiogalactopyranoside (IPTG) to a concentration of 1 mM, and protein was expressed overnight at 18° C. Cells were pelleted by centrifugation and resuspended in a small amount of PBS. After lysis using the Emulsiflex system, the sample was pelleted by centrifugation and the supernatant which contains the Lov fragment was bound to a glutathione column. The Lov portion of the fusion protein was released from the resin by overnight cleavage with PreScission Protease (GE Life Sciences). Centricon filter columns (Amicon) were then used to concentrate the fusion protein and remove any impurities.

#### Affinity Purification of the Lov antibodies

To affinity purify the Lov antibodies, the purified Lov fragment with the GST tag removed was cross-linked to NHS-activated Sepharose resin (GE Life Sciences) following the manufacturer's protocol. The Lov antibodies were then passed over the resin, which was then extensively washed to remove any impurities. Finally, the purified antibodies were eluted with a low pH buffer (100 mM glycine, pH 2.8). The eluted fractions were immediately neutralized by the addition of a neutralization buffer (1 M Tris, pH 8.5). The purified antibodies were then tested on whole head extracts.

#### Embryo collection and staining

For embryo staining, embryos were collected using the "grape plate" collection system. Briefly, adult flies were placed in three-cornered plastic beaker and a 50 mm Petri dish filled grape juice/agar mixture (grape plate) and smeared with yeast was placed over the opening. The flies were then allowed to lay eggs for 24 hours. After 24 hours, the grape plate was removed and was replaced with a new grape plate. If defined

embryonic stages were desired, embryos were collected on grape plates for two hours, then aged to the desired stage. For examination of the embryonic nervous system, embryos were aged for 22 to 23 hours prior to immunostaining. Embryos were then collected from the plates and the egg shell, the chorion layer, was removed through chemical dechoriation with 50% bleach. Next, the embryos were fixed in a mixture of heptane and 4% paraformaldehyde. The paraformaldehyde layer was removed and 100% methanol was added. Tubes containing the embryos were then vigorously shaken to remove the vitelline layer of the embryo. A series of washes with methanol were then performed to remove all remaining heptane and paraformaldehyde.

For the immunostaining, the embryos were first rehydrated into PBS. They were then blocked in PBT plus 5% Normal Goat Serum for up to 24 hours. Next, the embryos were stained with the primary antibody (Affinity purified Guinea Pig anti-Lov at 1:400 and/or 22C10 at 1:200 dilution) overnight at 4° C. Excess primary antibody was washed away and anti-guineas pig secondary antibody was added, either tagged with biotin or alkaline phosphatase, and incubated for two hours at room temperature. The staining pattern was then detected in the following ways. If a secondary antibody tagged with biotin was used, avidin and biotin conjugated to HRP were added. Avidin has four biotin binding sites and thus will link the secondary antibody and the HRP and amplify at the same time. Diaminobenzidine (DAB) was then added, producing a brown-colored product. If a secondary antibody tagged with alkaline phosphatase was used, X-Phosphate and Nitroblue tetrazolium salt were added, producing a blue colored product. If a double immunostaining was performed, DAB was developed first, followed by alkaline phosphatase.

Immunostaining of  $lov^{66}$  embryos was modified due to the inability of  $lov^{66}$  homozygotes to produce progeny. A  $lov^{66}/CyO-GFP$  stock was used for egg collections. After collection, the embryos were first stained with a GFP antibody and developed with alkaline phosphatase following the protocol described above. Embryos which did not stain with GFP antibody were the  $lov^{66}$  homozygous embryos and were immunostained with the Lov antibody and developed with DAB.

## CHAPTER 5: lov APPEARS TO HAVE A ROLE IN DROSOPHILA FERTILITY

As mentioned in Chapter 3, one of the mutants generated during the transposon mutagenesis,  $lov^{66}$ , has a fertility defect. Homozygous adults could be generated, but when these homozygous adults were mated, they did not produce offspring. This apparent fertility defect could be due to either a molecular defect in gametogenesis as suggested by the predicted  $lov$  expression in the testes (Flyatlas.org) or a behavioral defect in courtship as suggested by the similarity of  $lov$  to fruitless. To determine the cause of the  $lov^{66}$  sterility, a series of experiments were performed. First, an analysis was performed to determine if the sterility is a male defect, a female defect, or both. Next, the function of the sperm and the ability of  $lov^{66}$  female to receive, store, and use the sperm were examined. Finally, courtship was examined to determine if males could initiate courtship and copulate.

Analysis reveals that  $lov^{66}$  is sterile when sib-mated but not when mated to wild type

A series of crosses to test fertility were performed to determine the source of the  $lov^{66}$  sterility. By crossing  $lov^{66}$  males and females to each other and to a control line, it could be determined if the  $lov^{66}$  sterility was due to a male factor, female factor, or both. The sterility crosses performed are summarized in Table 5-1. The total number of progeny a given line could produce was determined by mating single males with single females for 24 days. After the initial eight days, the pairs were transferred to a new vial to prevent from mating with offspring and allowed to mate for another eight days. They were then transferred to a third vial and allowed to mate for another eight days at which point they are discarded. The vials are then watched for eclosion of adult offspring and the number of offspring from each vial was then counted for up to eight days after the

Cross	Male	Female
S1	w <sup>1118</sup>	lov <sup>66</sup> /lov <sup>66</sup>
S2	lov <sup>66</sup> /lov <sup>66</sup>	w <sup>1118</sup>
S3	lov <sup>66</sup> /lov <sup>66</sup>	lov <sup>66</sup> /lov <sup>66</sup>
S4	w <sup>1118</sup>	lov <sup>66</sup> /CyO
S5	lov <sup>66</sup> /CyO	w <sup>1118</sup>
S6	lov <sup>66</sup> /CyO	lov <sup>66</sup> /CyO
S7	w <sup>1118</sup>	w <sup>1118</sup>

Table 5-1: Mating Pairs for the Analysis of lov<sup>66</sup> Fertility. The first set of crosses, S1-S3, examined the effect of the homozygous mutation on fertility. The second set of crosses, S4-S6, examine the fertility of the heterozygous mutants and serve as a control for the homozygous mutants. The last cross, S7, determines the number of offspring produced by control flies.

initial eclosion.

These tests showed that when female  $lov^{66}/lov^{66}$  mutants are mated with wild type males (S1) there is a decrease in the progeny number produced as compared to:

i.)  $lov^{66}/CyO$  heterozygous females mated with control ( $w^{1118}$ ) males (S4); ii.)  $lov^{66}/lov^{66}$  males mated with control females wild type (S2); or iii.) control sib-matings (S7) (Figure 5-1).  $lov^{66}/lov^{66}$  males mated with control females (S2) did not show a decrease in progeny number as compared to  $lov^{66}/CyO$  heterozygous males mated with control females (S5) or control sib-matings.

When the number of progeny produced in the  $lov^{66}$  homozygous and heterozygous sib-mating (S3 and S6) is examined, it is seen that there is a slight decrease in the number of offspring produced when  $lov^{66}/CyO$  males are mated to  $lov^{66}/CyO$  females (S5) compared to control sib-mating (S7). However when  $lov^{66}/lov^{66}$  males are mated to  $lov^{66}/lov^{66}$  females (S3), on average, less than one offspring is produced per mating pair.  $lov^{66}/lov^{66}$  males mated with control females (S2) did not show a significant decrease in progeny but  $lov^{66}/lov^{66}$  females mated with control males showed a significant decrease in progeny number, it is apparent that the female mutant contributes more than male mutant to the observed decrease in fertility in the sib-mate cross of  $lov^{66}/lov^{66}$  mutants.

Use of a don-juan-GFP (dj8-GFP) fusion construct to examine spermatogenesis and sperm storage in  $lov^{66}$  mutant animals.

Microarray data suggests that *lov* expression is the highest in the head (brain) and in the testes (Flyatlas.org). Therefore, it seemed possible at first that the observed fertility defect in the  $lov^{66}$  mutants might be due to a defect in spermatogenesis, such as failure in

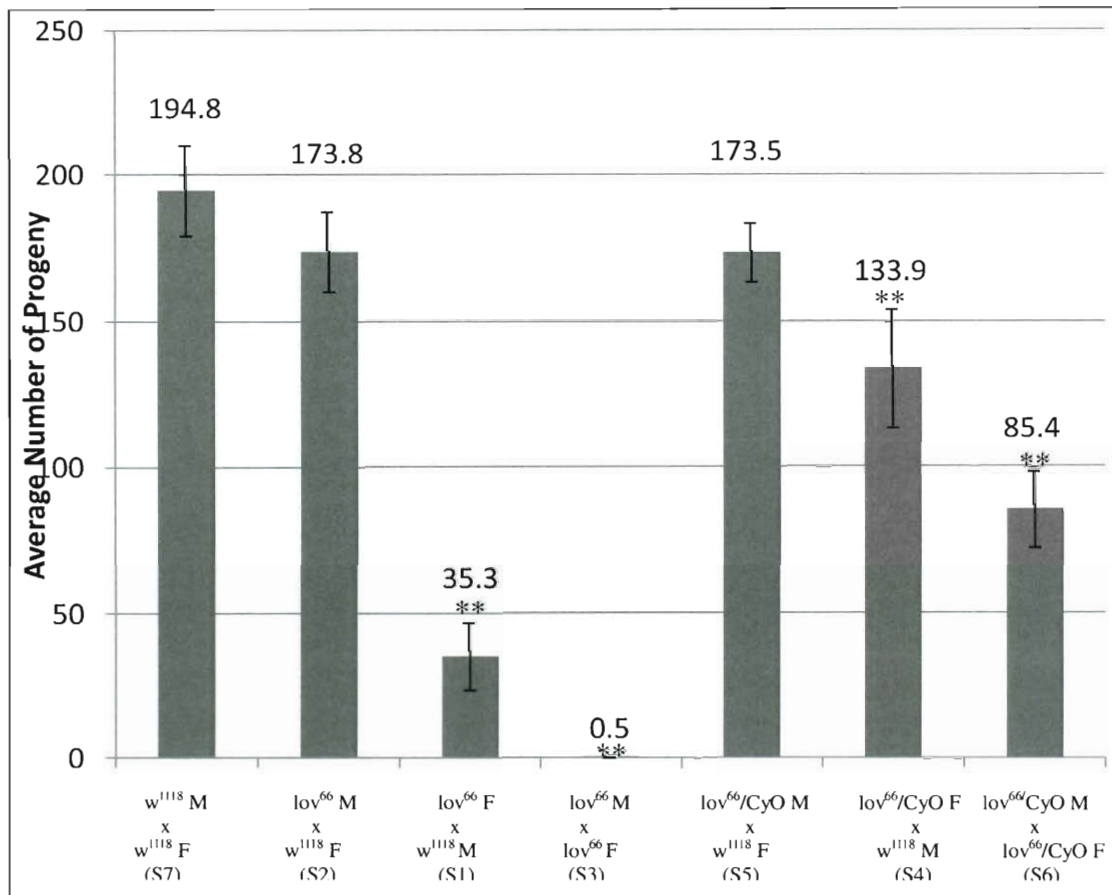


Figure 5-1: Results from fertility analysis lov<sup>66</sup>. The decreased fertility observed in the lov<sup>66</sup> homozygous pair mating is attributed to the decreased fertility of the female mutant. Female lov<sup>66</sup> mutants have significantly decreased fertility when compared to lov<sup>66</sup> males in terms of producing viable progeny when mated to control flies. A one-way ANOVA was performed to determine if there a difference between the different pairing. A Dunnett's Test was performed with w<sup>1118</sup> as the control. (M=males; F=female) (  $p \leq 0.001$ ; S1 n=23; S2 n=16; S3 n= 13; S4 n= 10; S5 n= 15; S6 n= 14)



generation of sperm or loss of sperm motility. However, based on the fertility analysis discussed above, it would appear that any defect in spermatogenesis would be a minor contributing factor to the  $lov^{66}$  sterility, given that  $lov^{66}$  males when mated to control females produce similar number of progeny as control sib-matings. Further, the fertility assay demonstrates that the sterility defect is primarily a female problem.

One possibility is that  $lov^{66}$  females may have defects in the acceptance and/or storage of sperm. To examine this aspect of fertility, the don-juan-GFP (dj8-GFP) construct was introduced into the  $lov^{66}$  genetic background (Figure 5-2). don-juan is normally expressed in the mitochondria in the sperm tail (Santel et al., 1998). The dj8-GFP construct will therefore express GFP in the sperm tail, allowing for the examination of sperm motility and morphology in males as well as sperm acceptance and storage of sperm in females.

Analysis of spermatogenesis in  $lov^{66}$  males reveals that  $lov^{66}$  produces functional sperm

The testes for three genotypes of males-dj8-GFP,  $lov^{66}/CyO$ -dj8-GFP, and  $lov^{66};dj8-GFP$  males- were dissected out, squashed, and examined with fluorescence microscopy. For dj8-GFP males,  $lov^{66}/CyO$ ;dj8-GFP males, and younger  $lov^{66};dj8-GFP$  males, most of the sperm were motile. When older  $lov^{66};dj8-GFP$  males were examined, most individuals had motile sperm, but in a small number of animals, the sperm were immotile and not properly individualized. Furthermore, sperm in the seminal vesicle were highly disorganized (Figure 5-3). To determine whether the defects observed in the older  $lov^{66};dj8-GFP$  males were not due to a genetic interaction of  $lov$  and dj8-GFP, the testes of  $w^{1118}$  and  $lov^{66}$  were examined by light microscopy alone. Between zero and three days old, both  $w^{1118}$  and  $lov^{66}$  produced motile sperm. After three days, in a small number of

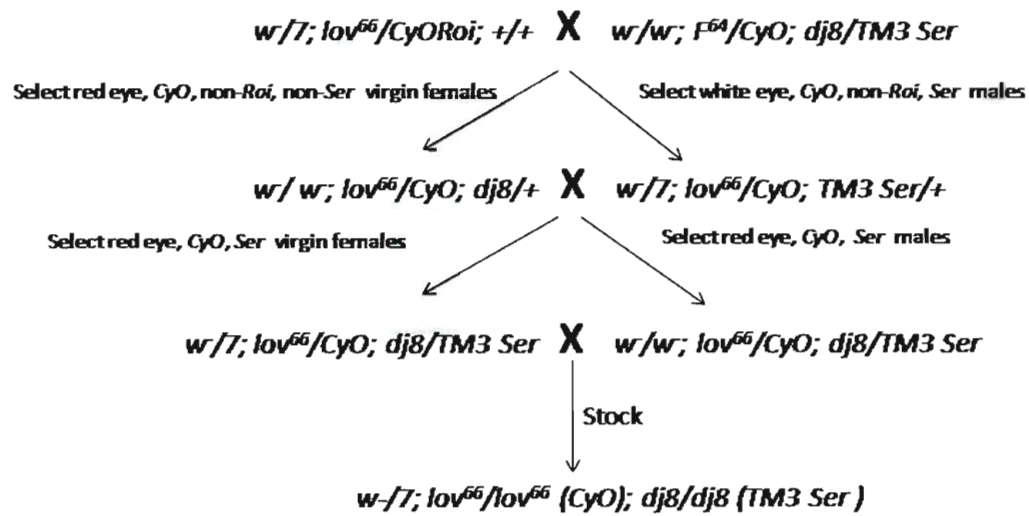


Figure 5-2: Crossing scheme for generating  $lov^{66}; dj8$ -GFP flies.

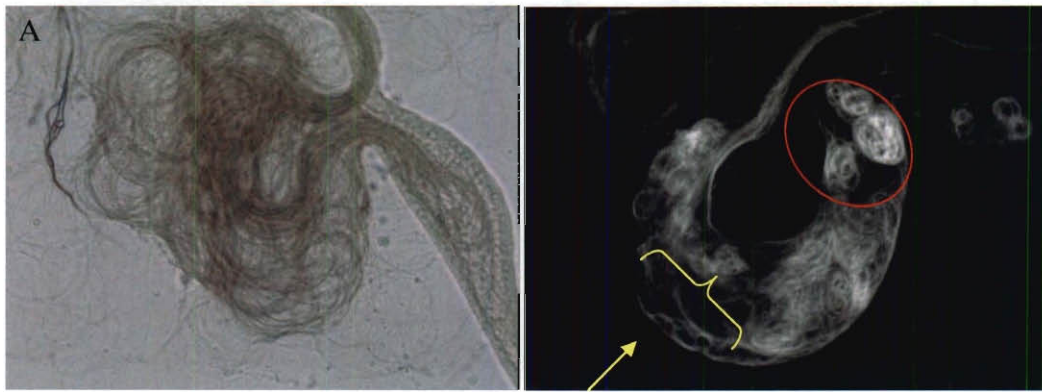


Figure 5-3: Defects in spermatogenesis are detected in the seminal vesicle of  $lov^{66}; dj8-GFP$  male. A. In the  $dj8-GFP$  males, the sperm found in the seminal vesicle are properly individualized and motile. B. In this  $lov^{66}; dj8-GFP$  male, the sperm which made it into the seminal vesicle appear to be highly disorganized and not properly individualized. The yellow arrow points to a region in which the sperm are properly individualized and motile. The red oval highlights three bundles of sperm which remained un-individualized.

lov<sup>66</sup> animals, however, there was a decrease in sperm motility accompanied by improper coiling of sperm, and coiling of un-individualized sperm. All three of these defects appeared to be enhanced by the dj8-GFP construct. This age related decrease in fertility was not observed in w<sup>1118</sup>. It would appear, then, that most lov<sup>66</sup> males are fertile but age-dependent defects appear. Because females are able to store a large amount of sperm for long periods, the initial mating with a lov<sup>66</sup> male would provide ample sperm for large numbers of progeny. Therefore, although there is a minor defect in lov<sup>66</sup> spermatogenesis, it probably makes a minor contribution to the sterility observed in lov<sup>66</sup>. Analysis of sperm acceptance and storage in lov<sup>66</sup> females reveals that lov<sup>66</sup> females can accept and store sperm.

A surprising result from the analysis of sterility was that female lov<sup>66</sup> mutants mated with male w<sup>1118</sup> produced 5- fold fewer viable offspring than lov<sup>66</sup> males mated with female w<sup>1118</sup>. Based on microarray data, one of the tissues that is predicted to have the highest lov expression is the testes (Flyatlas.org). Therefore, it would have been expected that lov<sup>66</sup> males would be responsible for the sterility of the lov<sup>66</sup> stock. Additionally, the analysis of sterility study demonstrated that lov<sup>66</sup> sib-mates had significant reduction in offspring produced; suggesting that there is a defect in both the males and females and when the two are combined, the line is almost completely sterile. These defects may involve problems with sperm transfer and storage. It is possible that the male cannot transfer sperm to the female perfectly or that the lov<sup>66</sup> females are unable to accept sperm or store the sperm.

To address these possibilities, sperm transfer and storage were investigated. In the first experiment, the lov<sup>66</sup> males' ability to transfer sperm and the lov<sup>66</sup> females' ability to

accept and/or store sperm was analyzed. To accomplish this,  $lov^{66}$  females were mated to a line containing the dj8-GFP transgene or to  $lov^{66};dj8-GFP$  males. This allows for visualizing sperm storage in the females upon dissection. As controls, female  $lov^{66}/CyO$  and  $w^{1118}$  were also mated with dj8-GFP and  $lov^{66} dj8-GFP$ -males.

As can be seen from Table 5-2, there is no significant difference in the  $lov^{66}$  males' ability to transfer sperm as compared to dj8-GFP or  $lov^{66} CyO; dj8-GFP$ . Almost 70% of  $lov^{66}$  and  $lov^{66}/CyO$  females had sperm in the seminal receptacle. Additionally, the  $lov^{66}$  females appear to be able to accept and store sperm as well as either control females ( $w^{1118}$  or  $lov^{66}/CyO$ ) as evidenced by the similar frequency of sperm storage under every condition. This suggests that the observed decrease in fertility in the  $lov^{66}$  sib-mating is not due to a defect in the ability of the males to transfer sperm or in the ability of the females to accept and store the sperm.

Eggs laid by  $lov^{66}$  females have reduced hatch and fertilization rates

In the next set of experiments, the ability of the sperm to fertilize eggs, the ability of the females to use the sperm, and the viability of the embryos were examined. For these experiments,  $lov^{66}$  males and females were mated either to the control line ( $w^{1118}$ ) or sib-mated. As controls,  $w^{1118}$  and  $lov^{66}/CyO$  flies were sib-mated. The eggs were then collected and allowed to age for 24 hours. Hatch rates were determined and those eggs that did not hatch after 24 hours were examined to determine whether or not the eggs had been fertilized. If most of the eggs in the  $lov^{66}$  sib-mating had been fertilized but never hatched, it suggests the  $lov^{66}$  mutation produces maternal effect embryonic lethalties.

	Male/Female Pairs				
	dj8-GFP M x w <sup>1118</sup> F	dj8-GFP M x lov <sup>66</sup> /CyO F	dj8-GFP M X lov <sup>66</sup> F	lov <sup>66</sup> -dj- GFP M X lov <sup>66</sup> /CyO F	lov <sup>66</sup> dj8- GFP M X lov <sup>66</sup> F
Storage in SR	10	12	12	9	10
No Storage in SR	3	6	3	4	5
n	13	18	15	13	15
Frequency of Storage	76.9%	66.7%	80.0%	69.2%	66.7%

Table 5-2: Sperm transfer and storage is not impaired in lov<sup>66</sup> mutants. To carry out this experiment, dj8-GFP, lov<sup>66</sup>/CyO; dj8-GFP, and lov<sup>66</sup>; dj8-GFP 3-6 days old males were individually mated with 3-6 days old virgin females, either lov<sup>66</sup>, lov<sup>66</sup>/CyO, or w<sup>1118</sup> for 6-9 days. The female sperm storage organs, seminal receptacles (SR) and spermathecae were dissected immediately after separation from the males and the presence of sperm in the seminal receptacles examined by fluorescence. Fluorescence of SR was then used to score for presence of stored GFP labeled sperm. There is no significant difference in lov<sup>66</sup> male ability to transfer sperm or in lov<sup>66</sup> female ability to store sperm when compared to w<sup>1118</sup> or lov<sup>66</sup>/CyO.

(n= number of SR examined;  $\chi^2=1.04$ )

On the other hand, if the majority of the eggs in the  $lov^{66}$  sib-mates are unfertilized, it suggests that the  $lov^{66}$  females are able to accept and store sperm but they are unable to use that sperm.

When the hatch rate was determined, it was found that  $lov^{66}$  females had lower hatch rates than the  $w^{1118}$  controls, whether they were mated to wild type males or sib-mated. The hatch rate for the  $lov^{66}$  females mated with  $lov^{66}$  males was 19.9% (n=1000 eggs) and 23.4% when  $lov^{66}$  females were mated with  $w^{1118}$  males (n=1600 eggs). The hatch rate for  $lov^{66}$  males mated with  $w^{1118}$  females was 65.6% (n=1100 eggs). The hatch rate for  $w^{1118}$  males mated with  $w^{1118}$  females was 77% (n=900 eggs) and the hatch rate for  $lov^{66}/CyO$  sib-mating was 88.5% (n=1300 eggs; hatch rate adjusted for presence of  $CyO/CyO$  eggs, which die just before hatching) (Figure 5-4). The sib-mated hatch rate is higher than expected, given that close to zero offspring were produced in the sib-mating (compare Figure 5-1). Therefore, it is possible that there is an incompletely penetrant larval lethality defect in  $lov^{66}$  that has not previously been observed and warrants further studies.

For the  $w^{1118}$  and  $lov^{66}$  sib-mating, those eggs that did not hatch were examined to determine if the eggs had been fertilized. When  $lov^{66}$  sib-mated unhatched eggs were examined, it was found that only about 25% of the eggs were fertilized (Figure 5-5). In comparison, about 80% of the unhatched  $w^{1118}$  eggs were fertilized.

The unhatched eggs were also examined for defects in morphology. While some of the eggs examined were normal in appearance, many had morphological defects. Some eggs were short and more round as opposed to ellipsoid in shape. There appears to be a continuum, from very round eggs to wild type appearing eggs. Eggs which have

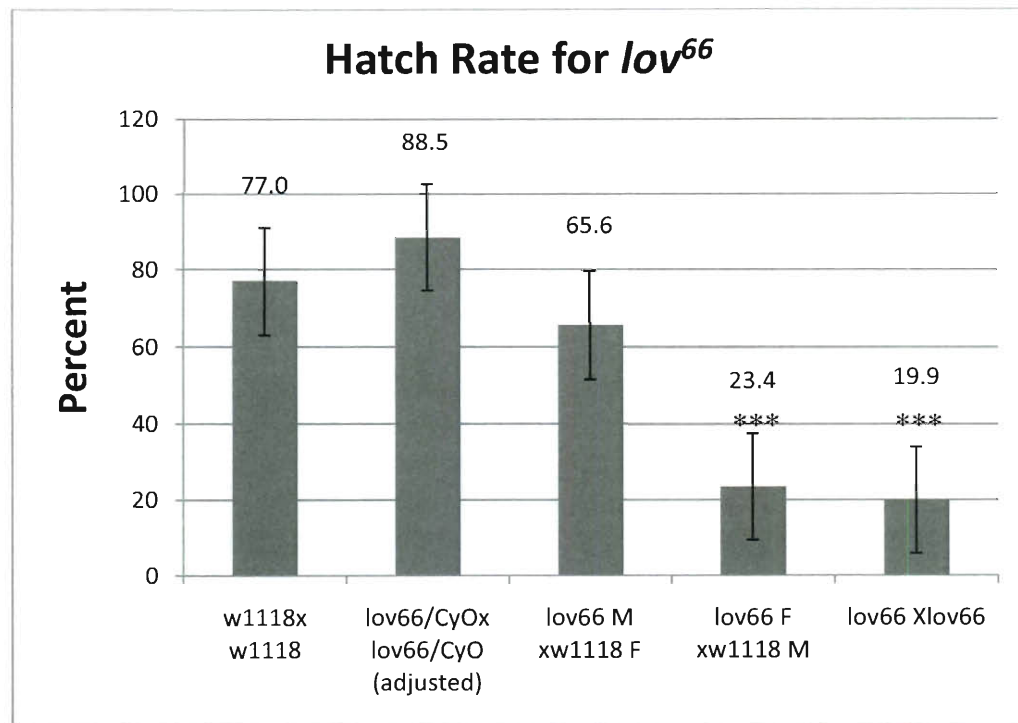


Figure 5-4: Homozygous  $lov^{66}$  females show decreased egg hatching rates. To carry out this set of experiments, three sets of 10-15 pairs of animals containing 3-6 days old males and similarly aged virgin females were collected and put together for mating in agar grape plates. After being placed together for one day, the animals were transferred into a new plate set-up. One hundred eggs which were laid on that first plate were transferred to a new plate. After 24 hours, the number of eggs that hatched was tallied. This experiment was repeated three times so at least 900 eggs from each set up were examined. When  $lov^{66}$  females are mated to either  $lov^{66}$  males or control ( $w^{1118}$ ) males, there is a significant decrease in the number of eggs hatching as compared to the sib-mating of the control ( $w^{1118}$ ) or  $lov^{66}/CyO$ .  $lov^{66}$  males had similar hatch rates as the control ( $w^{1118}$ ) or  $lov^{66}/CyO$  sib-mating when mated to control females. The hatch rate of for  $lov^{66}/CyO$  sib-mating was adjusted for the presence of  $CyO/CyO$  eggs (which die immediately before hatching) by adding 25%. A one-way ANOVA and a Dunnett's Test



were (Figure 5-4 cont.) performed to determine if there is statistical significant between the  $lov^{66}$  males and females hatch rates and the  $w^{1118}$  sib-mate hatch rate. ( $p \leq 0.001$ ;  $w^{1118}$  sib-mate  $n = 9$ ;  $lov^{66}/CyO$  sib-mate  $n = 13$ ;  $lov^{66} M \times w^{1118} F$   $n = 11$ ;  $lov^{66} F \times w^{1118} M$ ;  $n = 16$ ;  $lov^{66}$  sib-mating  $n = 10$ )

morphological defects may be unable to be fertilized, contributing to the low number of progeny produced by  $lov^{66}$  females.

Therefore, the observed decrease in fertility of  $lov^{66}$  appears to be due to several factors. First, the observed egg defect may make the eggs unfertilizable. Second, the females may be unable to use sperm effectively. Finally, there may be defects in oogenesis that then affect embryogenesis.

Analysis of courtship behavior in  $lov^{66}$  reveals courtship abnormalities.

In addition to the defects in spermatogenesis and oogenesis indicated above, it is possible, given the similarity between  $lov$  and  $fru$ , that  $lov^{66}$  may also produce courtship defects. To test for this, a number of courtship assays were performed on  $lov^{66}$  homozygous and heterozygous males, along with a control line, Canton S or Ore R. Isogenized  $lov^{66}$  homozygous and heterozygous males (discussed further in Chapter 6) were used to ensure that all mutations under study are in the same genetic environment.

The first courtship assay performed was to determine whether  $lov^{66}$  males courted wild type females. Naïve males were tested for courtship as described in Materials and Methods and the amount of time that the male spent actively courting the female was recorded. The Courtship Index (CI), which is the percentage of the time the male spends courting the female, was then determined. (Villella et al, 1997).

As can be seen from Figure 5-6, for both  $lov^{66}$  and  $lov^{66}/CyO$  males, there is a slight decrease in the Courtship Index. However this decrease is not statistically significant. Therefore, a defect in courtship is not responsible for any decrease in fertility when a  $lov^{66}$  male is paired with a wild type female. The above result is not surprising given that the number of offspring produced by  $lov^{66}$  males when mated to wild type

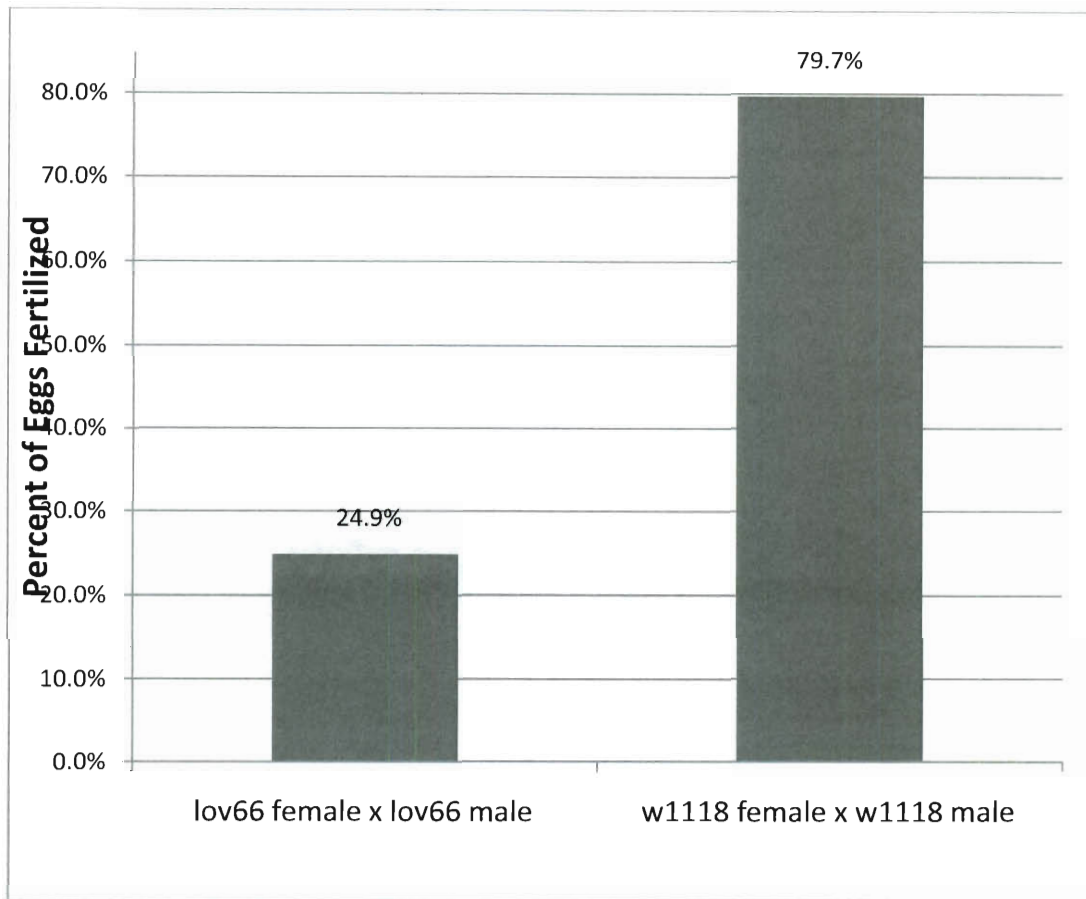


Figure 5-5: Homozygous  $lov^{66}$  females show decreased egg fertilization rates. The chorion layer (the outmost layer of the egg) was removed using chemical dechoriation. The eggs were then examined under a dissection microscope to determine if any development had occurred indicating that fertilization had occurred. When unhatched eggs from a  $lov^{66}$  sib-mate were examined, only about 25% proved to have been fertilized. This compared to about 80% fertilized in the control,  $w^{1118}$ . A Chi-squared test was performed to determine statistical significance. ( $\chi^2 = 65.9$ ;  $lov^{66}$  sib-mate  $n = 209$ ,  $w^{1118}$  sib-mate  $n = 69$ )

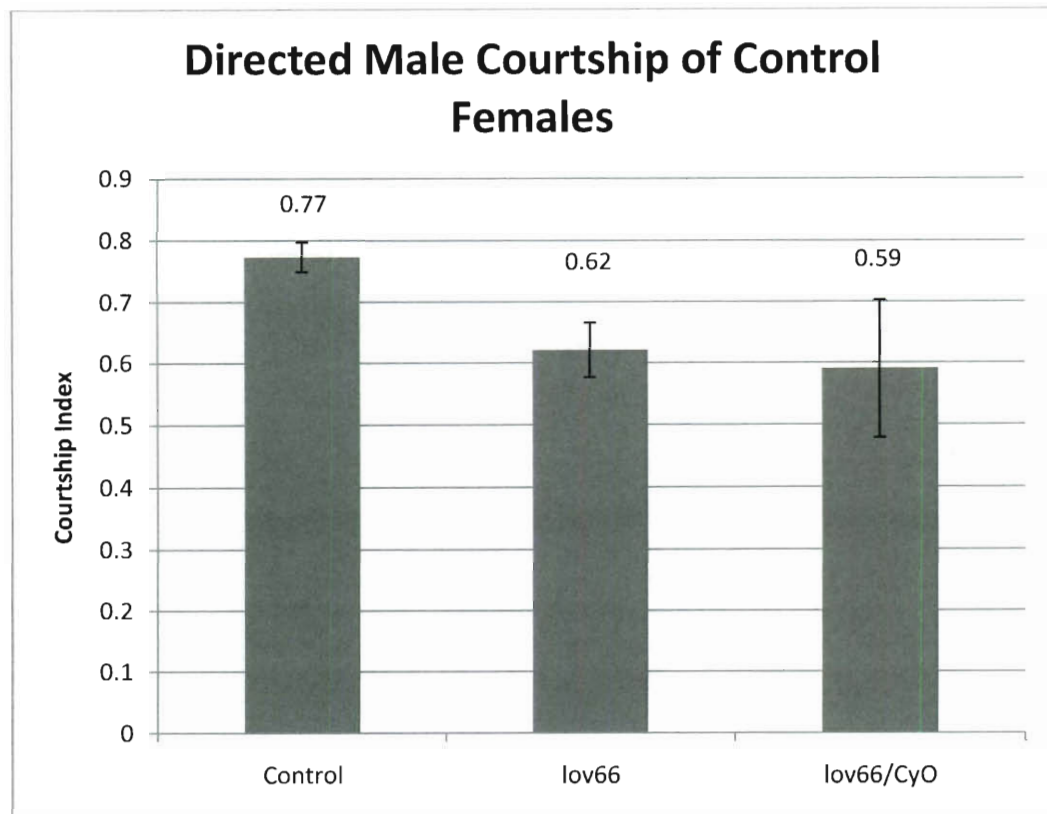


Figure 5-6: Courtship Index for  $lov^{66}$  and  $lov^{66}/CyO$  males with wild type females is indistinguishable from Canton S males. Naïve males were tested for courtship as described in Materials and Methods and the amount of time that the male spent actively courting the female was recorded. There is a slight decrease in the Courtship Index (the percentage of time the male spends courting the female) but it is not statistically significant.  $lov^{66}$  is able to court wild type females essentially as well as wild type males. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of  $lov^{66}$  and  $lov^{66}/CyO$  to the control line. ( $p=0.0647$ ; Control=Ore R and Canton S  $n=19$ ;  $lov^{66}$   $n=14$ ,  $lov^{66}/CyO$   $n=11$ )

females is high. However, when  $lov^{66}$  males and females are sib-mated, the number of offspring produced is greatly reduced. It is possible that the  $lov^{66}$  mutation makes the females poor targets for courtship or makes them unreceptive to males' courting attempts. Therefore, a courtship assay was performed in which  $lov^{66}$  females were the courtship target. For this assay, naïve males of three genotypes- Canton S,  $lov^{66}$ , and  $lov^{66}/CyO$ - were collected and assayed for their ability to court  $lov^{66}$  females (Figure 5-7).

As can be seen from Figure 5-7, for all three types of males, the Courtship Index is similar for  $lov^{66}$  females and wild type females. Although the Courtship Index for  $lov^{66}$  and  $lov^{66}/CyO$  males as compared to wild type males is slightly lower with either wild type females or  $lov^{66}$  females; this decrease is not statistically significant. Therefore, it would appear that  $lov^{66}$  females are good targets for courtship and  $lov^{66}$  males can court either almost as well as wild type.

It is possible although courtship proceeds normally, that the  $lov^{66}$  females ultimately refuse to mate with any male. Successful copulation is an indicator of whether females are receptive to male courtship attempts. When the males' ability to copulate with  $lov^{66}$  females was examined, it was found that males from all three lines were able to copulate with  $lov^{66}$  females. This suggests that  $lov^{66}$  females are receptive to the males' copulation attempts.

Given the similarities between *lov* and *fru* and that fact that mutations in *fru* cause male-male courtship phenotype, it is possible that  $lov^{66}$  males indiscriminately court both males and females. As a result, the observed decrease in fertility could be due to a reduction in time males spend courting and copulating with females because they are

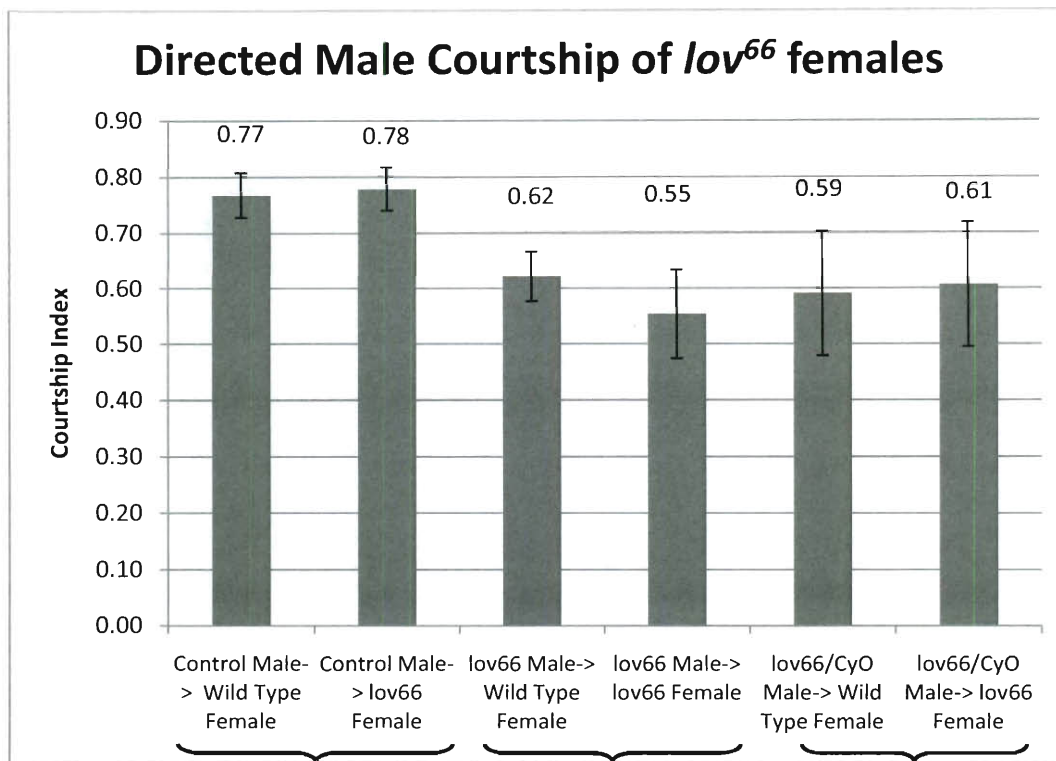


Figure 5-7: Courtship Indices for *lov<sup>66</sup>* and *lov<sup>66</sup>/CyO* males when paired with either wild type or *lov<sup>66</sup>* females. In this second courtship assay, the ability of *lov<sup>66</sup>* and *lov<sup>66</sup>/CyO* males to court *lov<sup>66</sup>* females versus wild type, Ore R, females was examined. Naïve males were tested for courtship as described in Materials and Methods with the exception that *lov<sup>66</sup>* were used. The amount of time that male spent actively courting the female was recorded and the Courtship Index was determined. For all three lines tested, there is no significant difference in courtship of wild type and *lov<sup>66</sup>* females. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line (Combined wild type males courting wild type females). A T-test was performed to compare males courting wild type female versus males courting *lov<sup>66</sup>* females. ( $p=0.261$ ; With Ore R females: Control=Ore R and Canton S  $n=19$ ; *lov<sup>66</sup>*  $n=14$ , *lov<sup>66</sup>/CyO*  $n=11$ ; With *lov<sup>66</sup>* females: Control=Canton S  $n=10$  ; *lov<sup>66</sup>*  $n=10$ , *lov<sup>66</sup>/CyO*  $n=10$ )

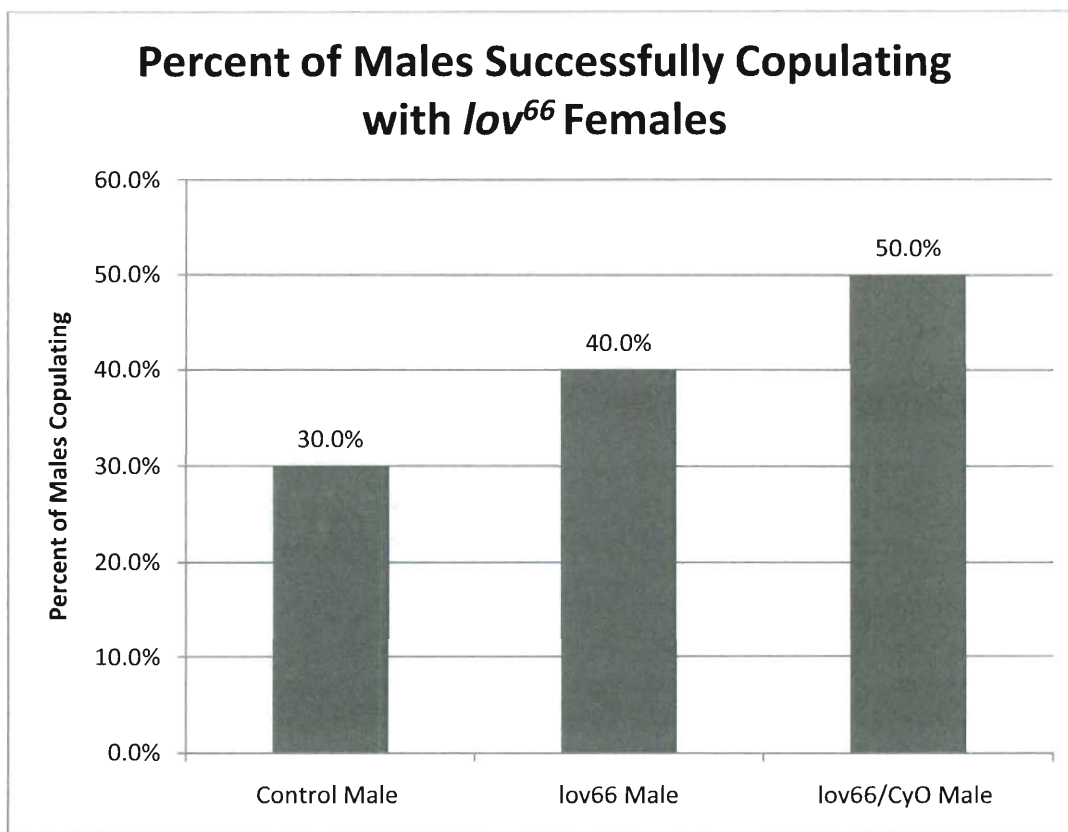


Figure 5-8: Percent of Successful Copulation of Canton S, *lov*<sup>66</sup>, and *lov*<sup>66</sup>/CyO when paired with either wild type or *lov*<sup>66</sup> females. For all three lines, males were able to copulate *lov*<sup>66</sup> females. This suggests that *lov*<sup>66</sup> females are receptive to the males' copulation attempts. (Control=Canton S n= 10; *lov*<sup>66</sup> n= 10, *lov*<sup>66</sup>/CyO n= 10)

courting other males. To test for this possibility, a male-male courtship assay was performed. In this assay, naïve males from the three genotypes- Ore R,  $lov^{66}$ , and  $lov^{66}/CyO$ - were collected and assayed for their ability to court wild type males (Figure 5-9).

It is clear from Figure 5-9, that  $lov^{66}$  males will court other males. To a certain extent, any male fly that is isolated for seven days will indiscriminately court males or females, which is reflected in the detectable male-male CI for the control and  $lov^{66}/CyO$  males. These two lines had similar male-male CIs, which are significantly lower than  $lov^{66}$  male CI values.

To address the question of whether the  $lov^{66}$  male observed sterility defect may be due, in part, to a reduction in the time  $lov^{66}$  males spend courting and copulating with females because they are courting other males, a competitive courtship assay was performed. With both a partner male and a partner female present, separate Courtship Indices were determined both for the male-female and male-male courtship.

When the male-female Courtship Index for the competitive courtship assay was determined for each line and compared to the non-competitive assay Courtship Index was, it the same or slightly lower (Figure 5-10). This indicates that when given the choice between courting a female versus another male, all three lines chose the female. The slight reduction in Courtship Index observed for  $lov^{66}$  and  $lov^{66}/CyO$  may be due to aggressive behavior shown towards the other male.

When the test male-target male Courtship Index in the competitive assay was determined and compared to the male-male non-competitive assay, it was found that for Canton S and  $lov^{66}/CyO$ , the Courtship Index was the same but for  $lov^{66}$ , there was a



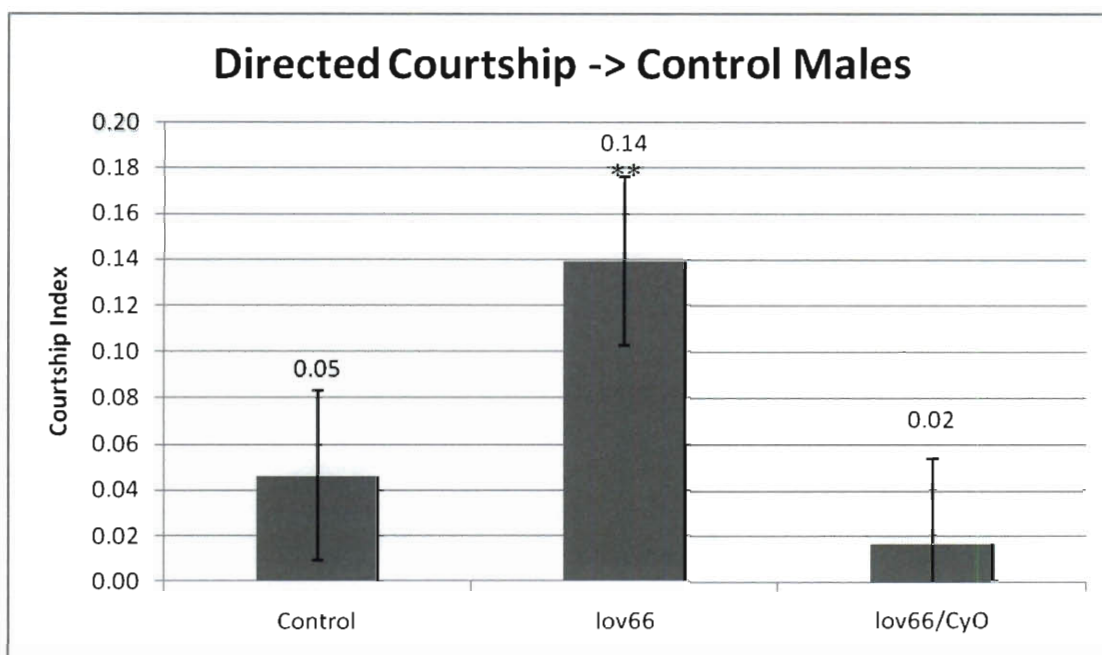


Figure 5-9: Courtship Index for  $lov^{66}$  and  $lov^{66}/CyO$  males when paired with control males. Naïve males were tested for courtship as described in Materials and Methods and the amount of time that the male spent actively courting the target male ( $w^+$ ; Sco/CyO) was recorded. The male-male Courtship Index was then determined. When  $lov^{66}$  males are paired with a control male, they spend more time courting the other male as compared to either the control male, Ore R, or  $lov^{66}/CyO$ . A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line. (  $p=0.006$ ; Control=Ore R  $n=10$ ;  $lov^{66}$   $n=13$ ,  $lov^{66}/CyO$   $n=10$ )

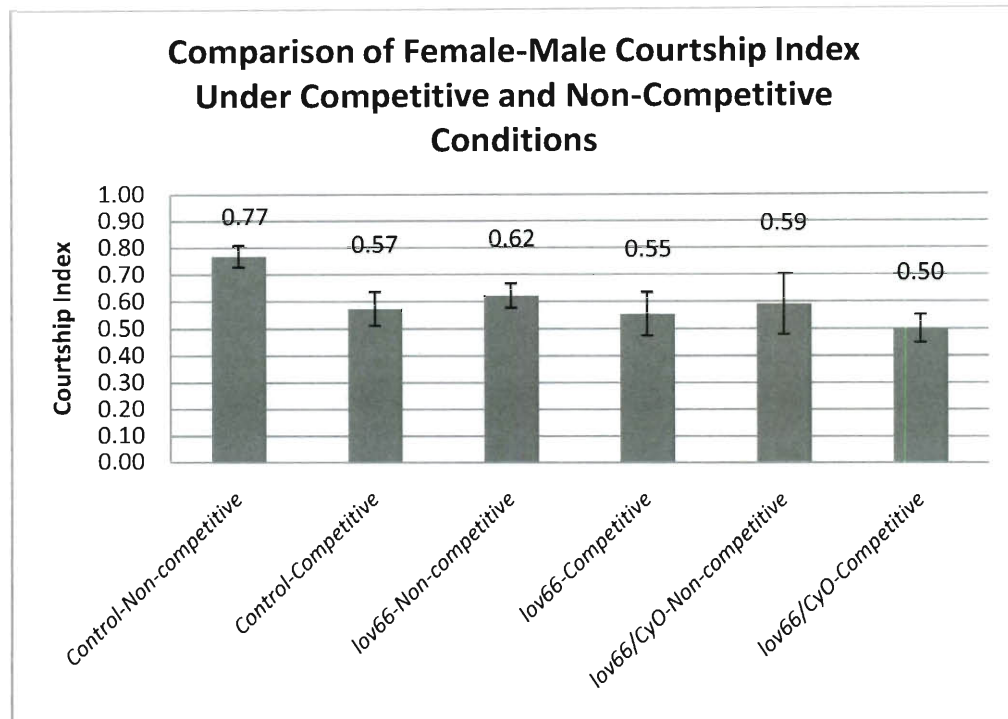


Figure 5-10: Comparison of male-female courtship in the non-competitive and competitive courtship assays. The courtship assay was performed as described in Materials and Methods with the exception that both a naïve control male ( $w^+; Sco/CyO$ ) and a virgin female were placed in the courtship chamber with the test male. The amount of time that the test male spent actively courting the female was recorded and the CI was determined. For the non-competitive and competitive conditions, all three lines had CIs that were similar or only slightly reduced, in a statically insignificant manner. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line (wild type males courting females under non-competitive conditions). A T-test between the non-competitive and non-competitive conditions was performed for each line. ( $p=0.276$ ; Non competitive Courtship: Control=Ore R and Canton S  $n=19$ ;  $lov^{66}$   $n=14$ ,  $lov^{66}/CyO$   $n=11$ ; Competitive: Control=Canton S  $n=10$ ,  $lov^{66}$   $n=10$ ,  $lov^{66}/CyO$   $n=10$ )

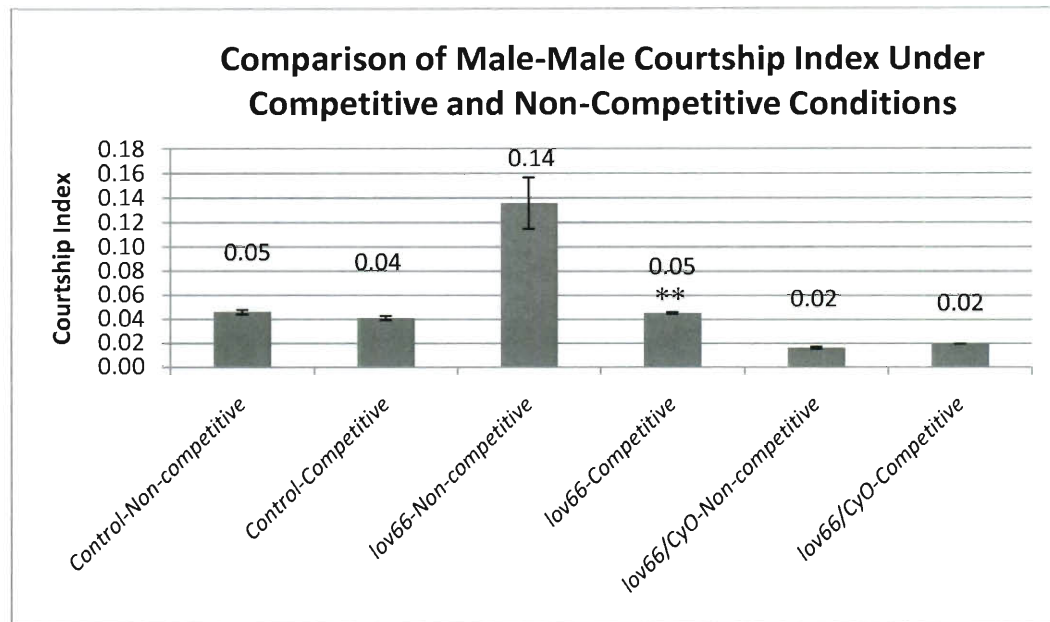


Figure 5-11: Comparison of male-male courtship in non-competitive and competitive assays. The courtship assay was performed as described in Materials and Methods with the exception that both a naïve control male ( $w^1; Sco/CyO$ ) and a virgin female were placed in the courtship chamber with the test male. The amount of time that the test male spent actively courting the target male was recorded and the C I was determined. For Canton S and  $lov^{66}/CyO$ , the CI was the same for the competitive and non competitive courtship assays and for  $lov^{66}$ , there was a significant drop in the CI in the competitive courtship assay to a value comparable to control values. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line (wild type males courting males under non-competitive conditions). A T-test between the non-competitive and non-competitive conditions was performed for each line. ( $p=0.002$ ; Non competitive Courtship: Control=Ore R & Canton S  $n=19$ ;  $lov^{66}$   $n=14$ ,  $lov^{66}/CyO$   $n=11$ ; Competitive: Control=Canton S  $n=10$ ;  $lov^{66}$   $n=10$ ,  $lov^{66}/CyO$   $n=10$ )

significant drop in the Courtship Index (Figure 5-11) to a value similar to control male-male courtship. Thus, when given the choice between courting females or other males,  $lov^{66}$  males will choose females.

#### Summary on findings

Based on the above behavioral studies, it is clear that the reduction in fertility observed in  $lov^{66}$  is not due to altered courtship behavior.  $lov^{66}$  males court females almost as well as control males.  $lov^{66}$  females appear to be appropriate courtship targets and are receptive to both control and  $lov^{66}$  male courtship attempts. Finally, while  $lov^{66}$  males will court other males, when given the choice, they will court females over other males.

Similarly, the defects in spermatogenesis do not appear to contribute significantly to the  $lov^{66}$  sterility defects. Only a few individual males appear to have defect a spermatogenesis, in which there is reduced sperm motility, improper coiling of the sperm, and incomplete individualization of the sperm. Rather, it appears that  $lov^{66}$  sterility defects may be largely due to defects in females and these defects may entirely be due to defects in oogenesis. The eggs appear to have morphological deformations, in which some of the eggs are more round than ellipsoid in shape. Finally, the females may be unable to use sperm to fertilize eggs, possibly due to the egg malformation.

## Material and Methods

### Courtship Assays

Naïve males were collected and allowed to age seven days in individual vials. They were then placed in courtship wheels with a partner, either 3-5 day old Control females (Ore R or Canton S) virgin females or 7-day old males ( $w^+$ ; Sco/CyO) aged in individual vials. Each pair was recorded for 10 minutes and the amount of time a male courted its partner was determined (O'Dell, 2003). That time was then used to calculate the Courtship Index (CI), which is the percentage of the time the male spends courting the female (Villella et al, 1997).

### Statistical Analysis

For each courtship assay, a one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line. A T-test was performed to determine if there is a difference for a line under different conditions. The test used for each set of experiments is stated in the figure for each experiment. An alpha of 0.05 was used for all statistics. The number of males tested (n) and the p-value is listed in each figure legend. Statistical significance is indicated in each figure in the following way: \* =  $p \leq 0.05$ ; \*\* =  $p \leq 0.01$ ; \*\*\* =  $p \leq 0.001$ .

## CHAPTER 6: EXAMINING COURTSHIP BEHAVIOR IN THE *lov* MUTANTS

As discussed in Chapter 5, one of the *lov* mutants generated in the transposase mutagenesis screen, *lov*<sup>66</sup>, has a fertility defect largely due to defects in females. These defects may entirely be due to defects in oogenesis as the eggs appear to have morphological deformations. Additionally, the females may be unable to use sperm to fertilize eggs, possibly due to egg malformation. Defects were also identified in spermatogenesis, but these defects are minor contributors to the overall sterility of *lov*<sup>66</sup>. However, given homology of *lov* and fruitless and our finding of minor courtship defects for *lov*<sup>66</sup>, it seemed possible that a greater role in courtship might be revealed by examining the other *lov* mutants. Therefore, the effects of the all *lov* mutations on courtship were examined. To control for factors outside the *lov* locus contributing to any observed courtship defects, a number of control experiments were performed.

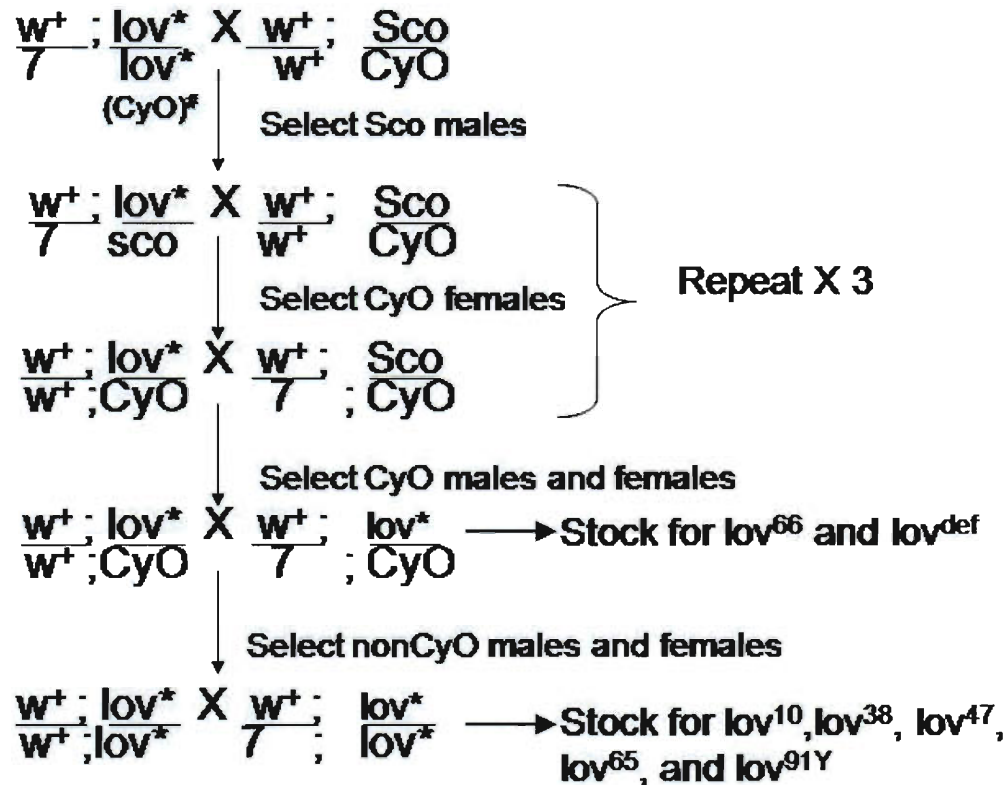
### Placing the *lov* mutants in an isogenic background

One of the concerns in behavioral studies is that differences in the genetic backgrounds of the mutations being studied may influence the behavior in question. To address this possibility, crosses are performed to ensure that the mutations under study are in the same genetic environment. Crosses were therefore undertaken to isogenize the other chromosomes of the *lov* mutant strains. The mutants were all crossed six times to a dominantly marked second chromosome line (Sco and CyO) and in which the other chromosomes were wild-type (Figure 6-1). The Sco and CyO markers allowed selection of the *lov* mutant chromosome while replacing the other chromosomes with wild-type chromosomes thus removing any other possible mutations. The outcrosses also placed the mutations in a wild type white (*w*<sup>+</sup>) eye color background. The original mutant lines were

in a mutant white ( $w^-$ ) eye color background. Previous behavioral studies have demonstrated that the  $w^-$  eye color background affects behaviors as the white gene encodes for a protein that transports both the eye pigment and neurotransmitters across membranes (Guen et al., 2000). Placing the flies in a  $w^+$  eye color background removes the possibility that any behavioral defect observed could be due to, at least in part, the  $w^-$  background. In addition to the five *lov* mutant lines *lov*<sup>10</sup>, *lov*<sup>38</sup>, *lov*<sup>47</sup>, *lov*<sup>65</sup>, and *lov*<sup>66</sup>, outcrosses were performed for 91Y and *lov*<sup>def</sup> to generate lines that will serve as control lines with the same genetic background as the mutants. The *lov*<sup>def</sup> line has the region of the chromosome around the *lov* locus removed but the rest of the chromosome is wild-type. It is used to rule out the possibility that a second mutation on the same chromosome as *lov* could be responsible for any observed courtship defects. These lines were used for behavioral experiments except where noted.

Deficiency crosses to investigate *lov* mutations as the source of courtship defects

The crosses to place the *lov* mutations in an isogenic background ensured that chromosomes 1, 3, and 4 were identical in the various mutant lines. However, the *lov* mutations each remained on their original second chromosome raising the possibility that additional differences on the second chromosome could affect courtship behavior. To control for this possibility, the various *lov* mutants and their controls were crossed to the isogenized *lov*<sup>def</sup> line. The isogenized line was generated in the crosses mentioned above and the original *lov*<sup>def</sup> line was received from the Bloomington Stock center (stock number 4961). The deficiency removes the region of the chromosome around the *lov* locus but the rest of the chromosome is wild-type (Pauli et al., 1995, Duffy et al., 1996, Preiss et al., 1985, Cote et al., 1987, Ludwig et al., 1991, Gotwals and Fristrom, 1991,



\*For  $lov^{66}$  and  $lov^{def}$ , stock is balanced over CyO

Figure 6-1: Crossing scheme for placing lov mutants and controls into an isogenic background



Germeraad et al., 1992). The result is offspring hemizygous for the mutant allele of *lov*. Any unrelated mutation on the *lov*<sup>mutant</sup> chromosome would be compensated by the wild type allele on the deficiency chromosome (Figure 3-3). If the courtship defect is due to a mutation at the *lov* locus that reduces *Lov* expression, then *lov*<sup>mutant</sup>/*lov*<sup>def</sup> flies would have the same or similar courtship defect as the *lov*<sup>mutant</sup>/*lov*<sup>mutant</sup> flies. However, if the mutation at the *lov* locus results in ectopic expression of *lov*, then placing the *lov* mutation over the deficiency might restore normal courtship. In this case, it would be difficult to determine if the courtship defect is due to a mutation at the *lov* locus or outside the *lov* locus as neither would demonstrate a courtship defect. Additional experiments such as examining the *lov* mutant expression pattern and looking for ectopic expression would be needed. For each of the lines listed above, the courtship assay was performed on both the homozygous flies and the *lov*<sup>mutant</sup>/*lov*<sup>def</sup> flies .

#### The control lines used for courtship experiments

Four control lines were used for the various courtship assays. The first control line used was 91Y. This is the original mutant line identified in the gravitaxis screen and the starting line used in the mutagenesis to produce the various *lov* mutants. Like the various *lov* mutants, it was placed in an isogenic background using the crossing scheme in Figure 6-1. The ability of the original 91Y line to court is similar to wild type as determined by previous courtship assays done in conjunction with the original gravitaxis screen (Armstrong et al., 2005). Normal courtship was one criterion used to select mutants for further analysis of gravitaxis defects.

*lov*<sup>10</sup> and *lov*<sup>65</sup> were selected as precise control lines. These lines were generated in the same transposase mutagenesis as the *lov* mutant lines and were selected because

the entire P element insertion was removed and the genomic region adjacent to the insertion was intact. Initial PCR reactions and sequencing confirmed this finding. The end result was two lines in which the *lov* locus believed to be wild type had gone through the same crosses as the mutant lines resulting in similar backgrounds. Like the various *lov* mutant lines, these lines were placed into an isogenic background. Additional sequencing was then used to confirm that the genomic region was still intact and that there were no deletions or base pair changes. The second round of sequencing revealed that both lines have a small 16 base pair deletion 77 base pairs downstream of the insertion site, which is not present in the original 91Y chromosome. Although much smaller, this deletion deletes some of the same DNA as in *lov*<sup>66</sup>. Therefore, it is possible that these lines will have similar behavioral defects to *lov*<sup>66</sup>.

Finally, as described above, the *lov* deficiency line was used to test for mutations outside of the *lov* locus on the second chromosome. As with the other lines, the deficiency line was placed into an isogenic background. One concern about the deficiency line is the deficiency chromosome is marked with Bristle (Bl), which results in shorter than average bristles. As bristles are important sensory organs and may be important in courtship, it raised the possibility that any defective courtship observed in the *lov*<sup>mutant</sup>/*lov*<sup>def</sup> flies could be due to Bl rather than the *lov* mutations. To determine the extent to which Bl affects courtship, the *lov* deficiency flies were crossed to wild type and *lov*<sup>def</sup>/+ flies were tested to see what, if any, affect Bl had on courtship.

## Results of the Courtship Assays

### Initial Courtship Assays for the Original *lov*<sup>66</sup> and *lov*<sup>65</sup> Mutant Lines

Courtship assays performed for *lov*<sup>66</sup> prior to isogenization (see above) revealed a

potential role for *lov* in courtship. A number of courtship assays were performed on *lov*<sup>66</sup> homozygous and heterozygous lines, along with various control lines, 91Y, *w*<sup>1118</sup>, and *lov*<sup>65</sup>. Naïve males were tested for courtship as described in Materials and Methods. They were scored for three parameters: Time to initiate courtship, percent of males courting, and Courtship Index (CI), which is the percentage of the time the male spends courting the female (Villegla et al, 1997).

When the percentage of males courting was compared between homozygous *lov*<sup>66</sup> and the control lines, most of the males in the control lines courted wild type females (Figure 6-2). For *lov*<sup>66</sup> homozygotes, only half of the males showed interest in the females. When the Courtship Index (CI) was calculated for each line, there were several interesting results (Figure 6-3). First, mutations of the white (*w*) gene clearly affect the CI. Of the five lines that were tested, only 91Y has red eyes. It also has the highest CI. This makes sense as the *w* gene encodes a transporter protein that transports neural transmitters and is known to affect behavior (Armstrong et al., 2005). The remaining lines have much lower CI. *w*<sup>1118</sup>, *lov*<sup>65</sup>, and *lov*<sup>66</sup>/CyO have CIs ranging from 0.12 to 0.19. This suggests that at least part of *lov*<sup>66</sup> courting defects is due to the presence of the *w* mutation. However, *lov*<sup>66</sup> homozygous males nevertheless have a much lower CI of 0.06 than the *w* controls (CI= 0.19), suggesting that the mutant has a strong defect in courting and that this defect is partially responsible for the observed sterility. When the time to initiation of courtship was calculated, *w* again affected the results. 91Y initiates courtship in about 12 seconds (Figure 6-4). The *w* controls, *w*<sup>1118</sup> and *lov*<sup>65</sup>, initiate courtship in 48 and 60 seconds respectively. For both the *lov*<sup>66</sup> heterozygote and homozygote, the time to initiate courtship is much longer, 88 and 144 seconds

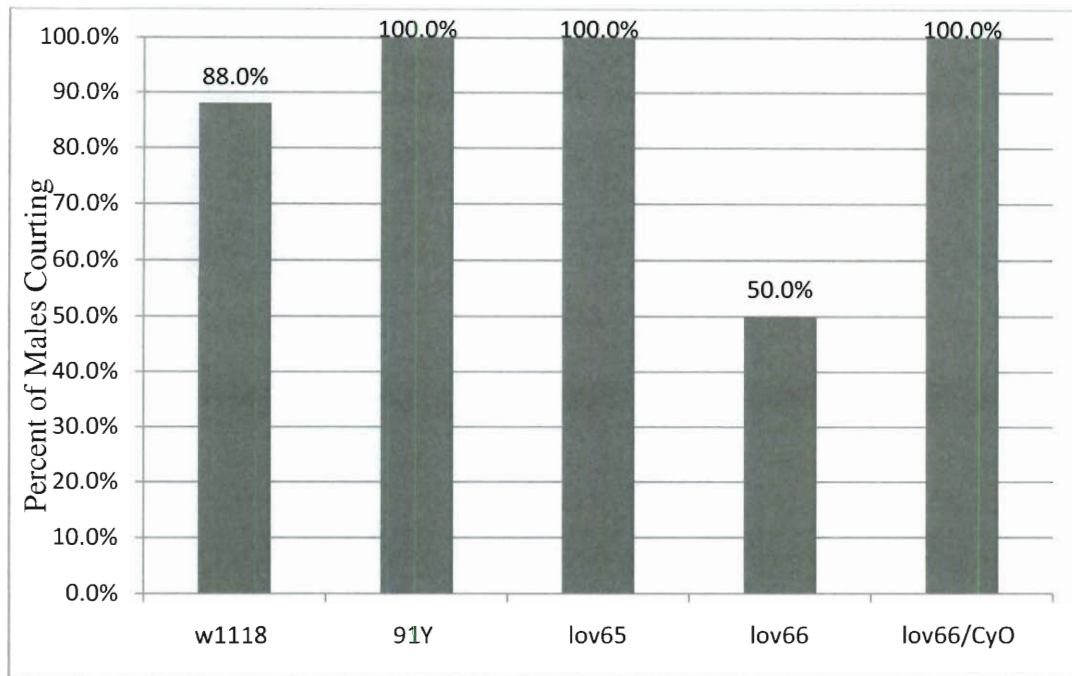


Figure 6-2: Percentage of flies that courted for various lov mutant lines. For every line except lov<sup>66</sup> homozygotes, most males courted wild type females. For lov<sup>66</sup> homozygotes, only half of the males showed interest in the females. (w<sup>1118</sup> n=10; 91Y n=10; lov<sup>65</sup> n=4; lov<sup>66</sup> n=8; lov<sup>66</sup>/CyO n=10)

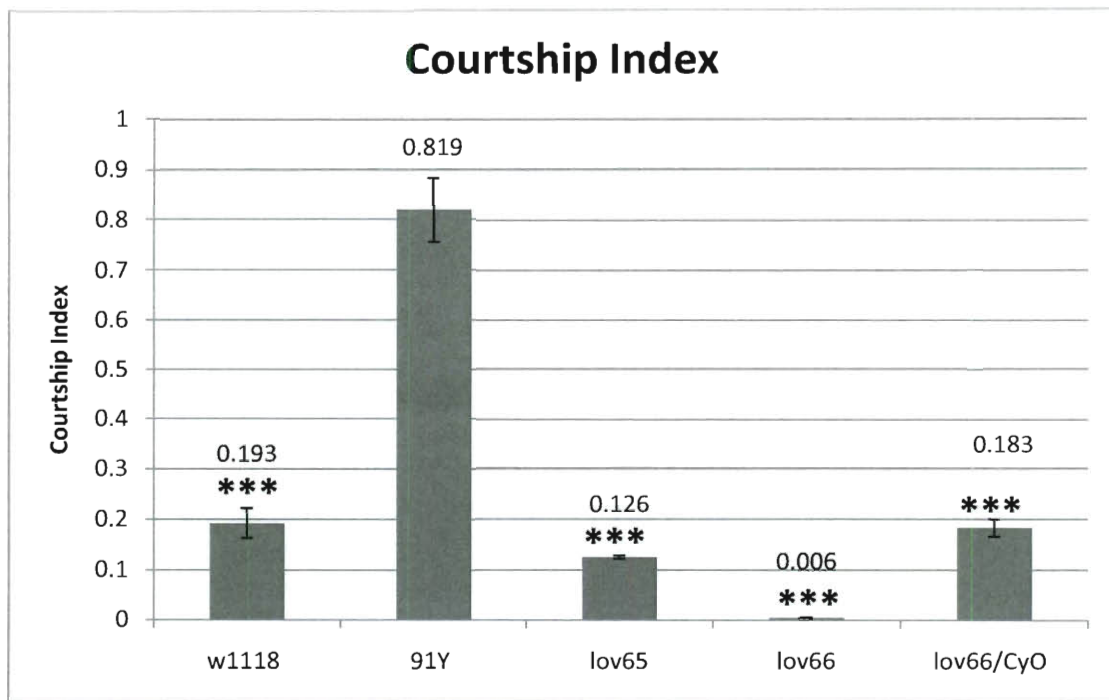


Figure 6- 3: Courtship Index(CI) of Various lov Lines. Red eyed flies (91Y) have a significantly higher CI than white eye flies. 91Y served as the red-eyed control and had a CI of 0.82.  $w^{1118}$  served as the  $w^-$  control and had a CI of 0.19.  $lov^{65}$  had a CI of 0.12.  $lov^{66}/CyO$  ( $lov^{66}$  heterozygote) had a CI of 0.18 which is similar to  $w^-$  wild-type, suggesting that the  $lov^{66}$  heterozygote has a similar ability to court as wild-type.  $lov^{66}$  homozygote had a much lower CI of 0.06, suggesting that the mutant has a strong defect in courting. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line, 91Y. ( $p \leq 0.001$ ;  $w^{1118}$   $n=10$ ; 91Y  $n=10$ ;  $lov^{65}$   $n=4$ ;  $lov^{66}$   $n=8$ ;  $lov^{66}/CyO$   $n=10$ )

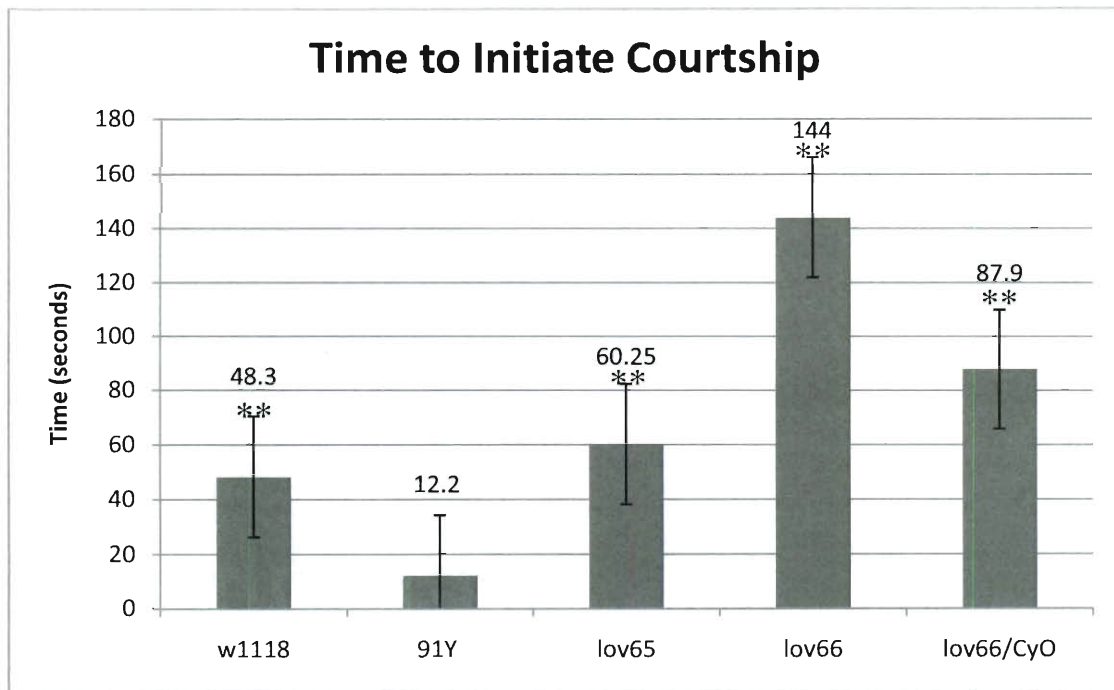


Figure 6-4: Time to Initiate Courtship of Various *lov* Lines. The red eye control, 91Y, initiated courtship in about 12 seconds. The white eye controls,  $w^{1118}$  and  $lov^{65}$ , initiated courtship in 48 and 60 seconds respectively. The time to initiate courtship for  $lov^{66}$  heterozygote and homozygote is 88 and 144 seconds respectively, much longer than any of the controls. All of the  $w^-$  lines took a statically significantly longer time to initiate courtship than the  $w^+$ , 91Y, control. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line, 91Y. (  $p=0.297$ ;  $w^{1118}$   $n=10$ ; 91Y  $n=10$ ;  $lov^{65}$   $n=4$ ;  $lov^{66}$   $n=8$ ;  $lov^{66}/CyO$   $n=10$ )

respectively.

While these initial results show that mutations in *lov* do cause a courtship defect, there is always the possibility that the observed defects are due to a mutation outside of the *lov* locus. Therefore, the courtship assays were repeated using the isogenized *lov* mutants lines. As before, naïve males were collected and allowed to age seven days. They were then placed in courtship wheels with 3 to 5 day old wild type virgin females and recorded for 10 minutes (O'Dell, 2003). Their Courtship Index was then determined.

When the *lov*<sup>66</sup> outcrossed line was tested, surprisingly the Courtship Index improved. The Courtship Index for the homozygote is 0.62. The Courtship Index for the heterozygote is 0.59 (Figure 6-5). These CIs are still below what is considered a normal CI of 0.8 to 1 but are not statistically significant. The improvement suggests that at least part of the courtship defect observed in the original lines is due to mutation outside of the *lov* locus, possibly due to the presence of white. Similar to *lov*<sup>66</sup> and *lov*<sup>66</sup>/*CyO*, the Courtship Index for *lov*<sup>65</sup> improved from 0.13 to 0.39 (Figure 6-5). This improvement is still significantly lower compared to wild type, however, suggesting that *lov*<sup>65</sup> has a courtship defect due to a mutation at the *lov* locus.

Surprisingly, the Courtship Index for 91Y decreased when it was placed in the isogenic background. The Courtship Index for the original mutant was 0.82 and decreased to 0.49 for the isogenized mutant (Figure 6-5). This suggests that there were mutations in original line that countered the effect of the insertional mutation of 91Y. Once any secondary mutations were removed, the courtship defect in 91Y was revealed.

The original *lov*<sup>10</sup>, *lov*<sup>38</sup> and *lov*<sup>47</sup> lines are fertile since homozygous stocks could be maintained and therefore were not initially tested for courtship defects.. However, the

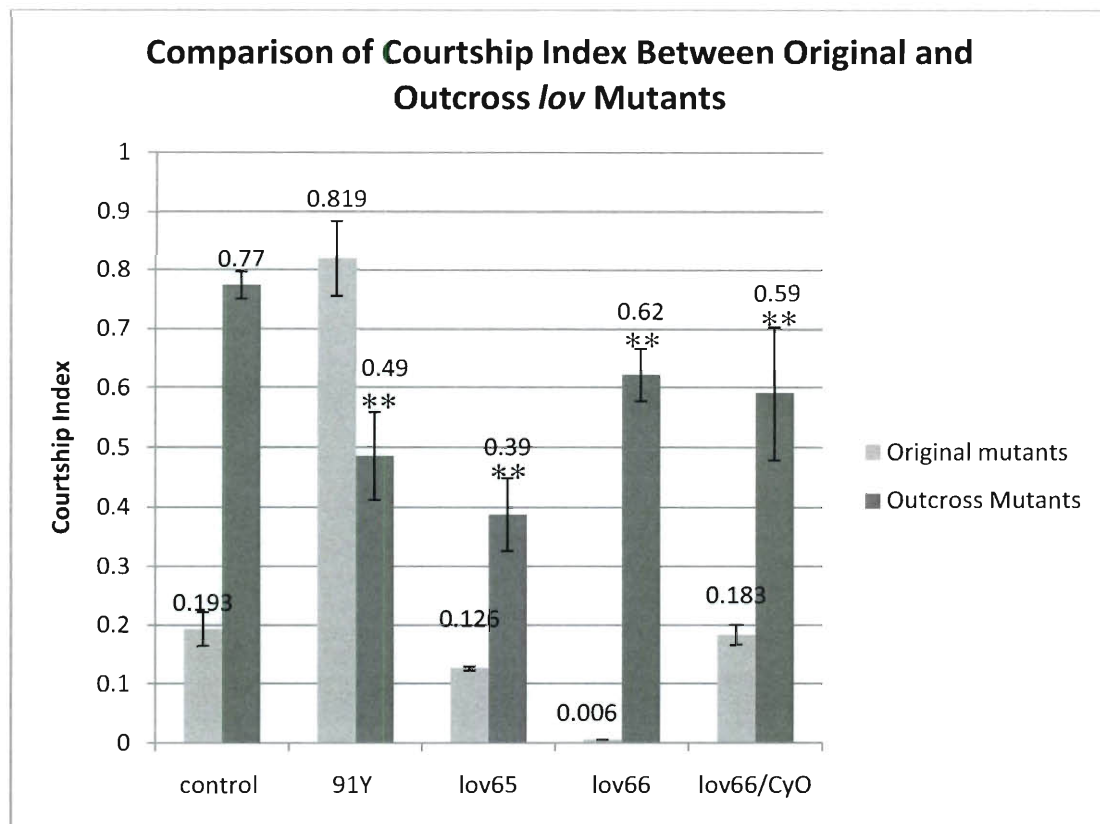


Figure 6-5: Comparison between original and isogenized *lov* mutants. The control for the original mutants was  $w^{1118}$  and the control of the outcross *lov* mutants was Ore R and Canton S (combined data). The controls were chosen such that the eye color matched the mutants. When the outcross  $lov^{66}$  has a higher CI improved than the original  $lov^{66}$  mutant. The CIs are still below what is considered a normal CI of 0.8 to 1 but not statistical significant. The CI for  $lov^{65}$  improved from 0.13 to 0.39, suggesting that  $lov^{65}$  has a courtship defect due to a mutation at the *lov* locus. It is also a statistically significant decrease compare to  $w^+$  control CI of 0.77. The CI for 91Y decreased when it was placed in the isogenic background. A T-test was performed for each pair, the original line and the isogenized line. ( $w^{1118}$  n=10; Ore R and Canton S n=19; 91Y original n=10; 91Y outcross n=10;  $lov^{65}$  original n=4;  $lov^{65}$  outcross n= 14;  $lov^{66}$  original n=8;  $lov^{66}$  outcross n=14;  $lov^{66}/CyO$  original n=10;  $lov^{66}/CyO$  outcross n=11)



isogenized lines were tested and they proved to have lower Courtship Indices than the wild type (Canton S and OreR) control lines. The Courtship Index for  $lov^{10}$  is 0.29. The CI for  $lov^{38}$  is 0.22. Finally, for  $lov^{47}$ , the Courtship Index is 0.26 (Figure 6-6). In addition all of the  $lov$  mutants demonstrated abnormal behavior in the courtship wheel. Several of the  $lov^{38}$  males attempted to copulate in the wrong location, either at the female's head or side or at the side of the courtship chamber. Additionally,  $lov^{38}$  and  $lov^{47}$  males appear to be sluggish in the courtship chamber. They primarily spend their time orientating towards the females whereas control males move through all the courtship steps. They make very few attempts at courting the female and often only attempt courtship when the female is in close proximity. The courting attempts typically only last as long as the females are in close proximity. Additionally,  $lov^{10}$  and  $lov^{65}$  appear to have trouble locating females and staying focused on females. Like  $lov^{38}$  and  $lov^{47}$  males, the males make few courting attempts, usually only when the female is in close proximity. Furthermore, the courting attempts only lasted while the female was in close proximity to the male and ended when the female moved away from the male. Unlike wild type males, none of the above  $lov$  mutants actively pursued the females.

Another unusual behavior observed in the above mutants is non-directed courtship. All four lines performed various courtship behaviors, most often wing extension, which appeared not to be directed towards the female. Many of the males in these lines wander around the chamber with one wing extended. This behavior was not observed in the control males or in  $lov^{66}$  or  $lov^{66}/CyO$  males.  $lov^{10}$  and  $lov^{47}$  have the highest levels of non-directed courtship with non-directed Courtship Indices of 0.073 and 0.098 (Figure 6-7). This behavior suggests that the males may know that there is a

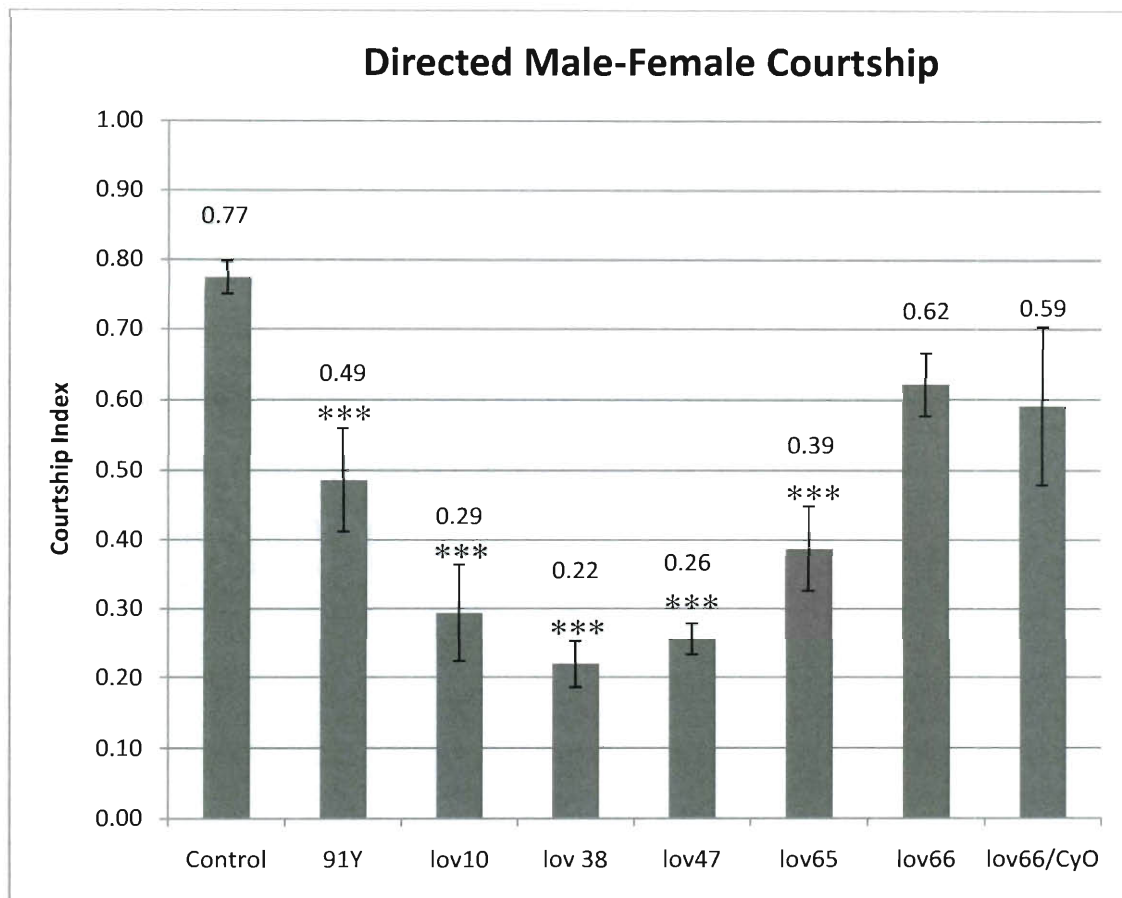


Figure 6-6: Directed male-female Courtship Indices for lov mutant lines. When the outcross lines were tested, they had a lower Courtship Index than the control line. For all the lines except lov<sup>66</sup> and lov/CyO, this decrease was statistically significant. In addition, all of the lov mutants demonstrated abnormal behavior in the courtship wheel. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control (Ore R and Canton S combined as the control). ( $p \leq .001$ ; Ore R and Canton S  $n=19$ ; 91Y  $n=10$ ; lov<sup>10</sup>  $n=13$ ; lov<sup>38</sup>  $n=19$ ; lov<sup>47</sup>  $n=10$ ; lov<sup>65</sup>  $n=14$ ; lov<sup>66</sup>  $n=14$ ; lov<sup>66</sup>/Cyo  $n=11$ )

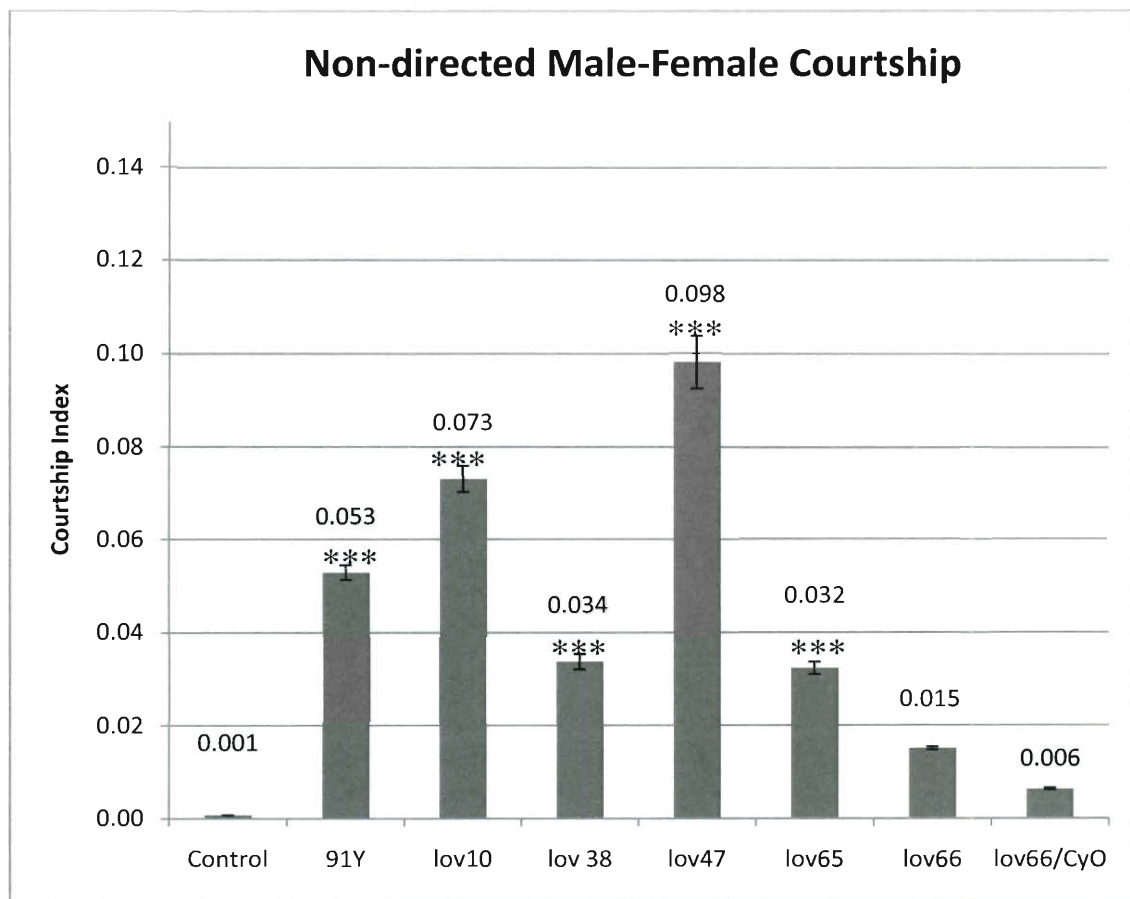


Figure 6-7: Nondirected male-female Courtship Indices of the *lov* mutant lines. 91Y, *lov*<sup>10</sup>, *lov*<sup>38</sup>, *lov*<sup>47</sup>, and *lov*<sup>65</sup> all performed various courtship behaviors, most often wing extension, which appeared not to be directed towards the female. Many of the males in these lines wander around the chamber with one wing extended. This behavior was not observed in the control males or in *lov*<sup>66</sup> or *lov*<sup>66</sup>/CyO males. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line (Ore R and Canton S combined as the control). ( $p \leq 0.001$ ; Ore R and Canton S  $n=19$ ; 91Y  $n=10$ ; *lov*<sup>10</sup>  $n=13$ ; *lov*<sup>38</sup>  $n=19$ ; *lov*<sup>47</sup>  $n=10$ ; *lov*<sup>65</sup>  $n=14$ ; *lov*<sup>66</sup>  $n=14$ ; *lov*<sup>66</sup>/CyO  $n=11$ )

female present in the chamber but have trouble identify the location of the female.

To confirm that the observed courtship defects were indeed due to the various *lov* mutations, each of the mutations was placed over the *lov* deficiency chromosome. As mentioned above, the result is offspring that only have one copy of *lov*, which is the mutant allele of *lov*. Any unrelated mutation on the *lov*<sup>mutant</sup> chromosome would be compensated by the wild type allele on the deficiency chromosome. If the courtship defect is due to a mutation at the *lov* locus that reduces the expression level, than *lov*<sup>mutant</sup>/*lov*<sup>def</sup> flies would have the same or similar courtship defect as the *lov*<sup>mutant</sup>*lov*<sup>mutant</sup> flies. However, if the mutation at the *lov* locus results in ectopic or over-expression of *lov*, then placing the *lov* mutation over the deficiency may restore normal courtship. To examine these possibilities, courtship assays were performed for the *lov*<sup>mutant</sup>/*lov*<sup>def</sup> as described in Material and Methods.

As a control, the Ore R, a wild type line, was crossed to the deficiency line as the deficiency line is marked with bristle (Bl), which results in shorter than average bristles. This was done to ensure that the Bl phenotype does not have an effect on courtship. As can be seen in Figure 6-8, the Ore R and Ore R/*lov*<sup>def</sup> have similar Courtship Indices. Therefore, any changes in courtship behavior in the *lov*<sup>mutant</sup>/*lov*<sup>def</sup> flies is not due to the presence of Bl.

For all of the *lov* mutants, the Courtship Index improves significantly when the mutant chromosome is placed over the deficiency chromosome. *lov*<sup>66</sup> and *lov*<sup>66</sup>/*def* have similar Courtship Indexes, both of which are not statistically significant from the control (Figure 6-8). This suggests that *lov*<sup>66</sup> does not have a courtship defect. When 91Y is placed over the deficiency, the Courtship Index increases from 0.49 to 0.64 (Figure 6-8).

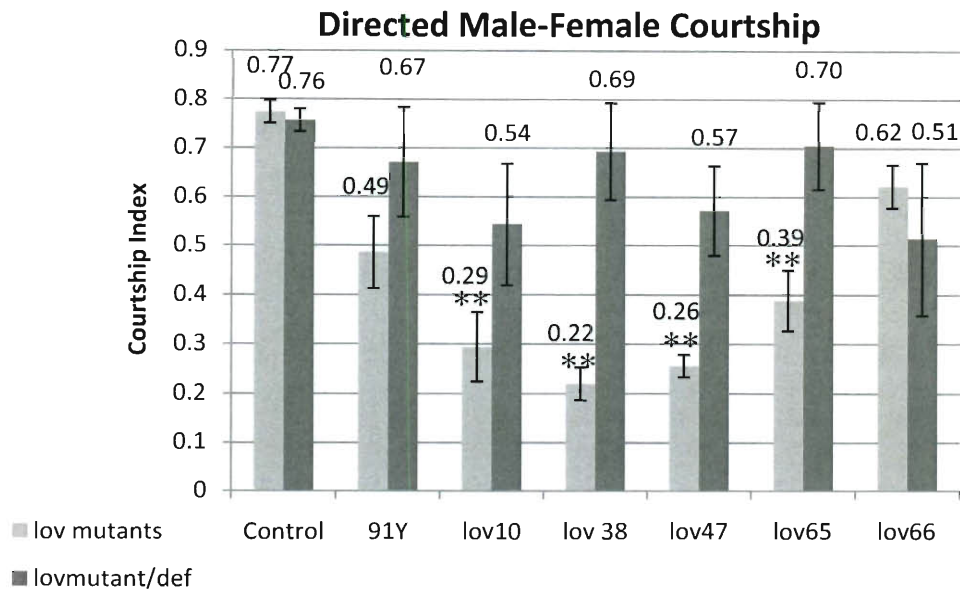


Figure 6-8: Comparison of Directed Courtship Between homozygous lov and hemizygous lov mutant in an isogenic background. When 91Y is placed over the deficiency, the CI increases but is not statistically significant.  $lov^{66}$  and  $lov^{66}/def$  have similar CIs, both of which are not statistically significant from the control, suggesting that  $lov^{66}$  does not have a courtship defect. For the remaining lov mutants, the CI is significantly higher when the mutant chromosomes are placed over the deficiency chromosome and is comparable to wild CI. A one-way ANOVA of the hemizygous mutants was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the hemizygous mutant lines to the control line (Ore R/def). No statistically significant difference was found between the hemizygous males. A T-test was then used to compare the original line to the line over deficiency, which identified statistically differences for (Figure 6-9 cont.)  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{47}$ , and  $lov^{65}$ . (p = 0.281; Ore R and Canton S n=19; OreR/def n= 7; 91Y n=10; 91Y/def n=22;  $lov^{10}$  n= 32;  $lov^{10}/def$  n= 22;  $lov^{38}$  n= 19;  $lov^{38}/def$  n= 26;  $lov^{47}$  n= 10;  $lov^{47}/def$  n= 10;  $lov^{65}$  n= 14;  $lov^{65}/def$  n= 20;  $lov^{66}$  n=14;  $lov^{66}/def$  n= 13)

This increase is not statistically significant however due to the wide variability of both 91Y and 91Y/lov<sup>def</sup>. For the remaining lov mutants, the CI increases when the mutant chromosomes are placed over the deficiency chromosome and this increase is statistically significant (Figure 6-8).

When non-directed courtship was examined in these flies, a similar pattern was observed. For lov<sup>38</sup>/lov<sup>def</sup>, lov<sup>65</sup>/lov<sup>def</sup>, and lov<sup>66</sup>/lov<sup>def</sup>, there was little or no change in the level of non-directed courtship (Figure 6-9). For 91Y, the non-directed courtship decreased from 0.05 to 0.03, but this decrease is not statistically significant. For lov<sup>10</sup> and lov<sup>47</sup>, there is a statistically significant decrease in the non-directed courtship. The non-directed courtship for lov<sup>10</sup> decreased from 0.07 to 0.02. The non-directed courtship decreased from 0.10 to 0.01 for lov<sup>47</sup>.

Based on the results of the deficiency courtship assays, it would appear that the courtship defects observed are not due to the mutations at the lov locus or that the lov<sup>def</sup> chromosome has been altered at the lov locus. To assess if the improvement is due to a problem with the lov<sup>def</sup> chromosome, two lines of analysis were performed. First, the lov<sup>def</sup> stock was examined to ensure that the lov<sup>def</sup> chromosome was still present. The lov<sup>def</sup> chromosome carries two easily identifiable markers that can be used to identify the chromosome. The first is Bl which causes a shortened bristle phenotype. The second marker is purple, which confers a purplish eye color to the flies. Flies from the isogenized lov<sup>def</sup> stock carry both of these markers, suggesting that the lov<sup>def</sup> chromosome is still present in the stock. Second, PCR was used to examine the deficiency chromosome and to assess if the lov locus has been removed. When PCR reactions are performed on lov<sup>66</sup> heterozygous flies using a primer pair adjacent to the insertion site,

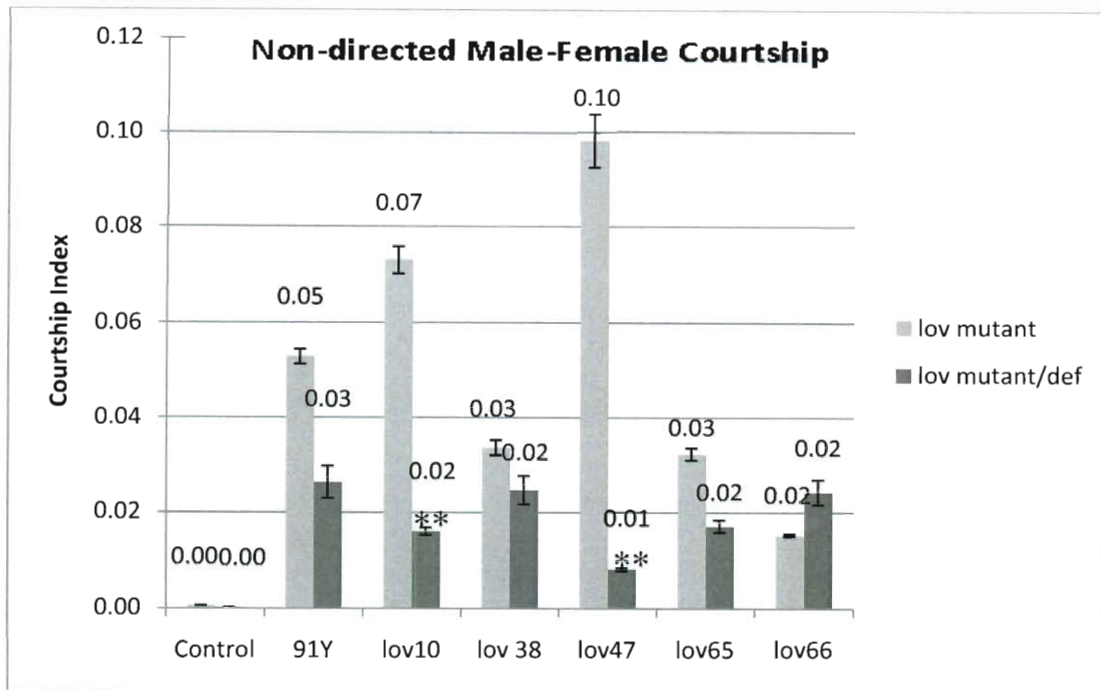


Figure 6-9: Comparison of Non-directed Courtship levels for homozygous lov mutants and hemizygous lov mutants. For  $lov^{38}/lov^{def}$ ,  $lov^{65}/lov^{def}$ , and  $lov^{66}/lov^{def}$ , there was little to no change in the level of non-directed courtship. For 91Y, the non-directed courtship decreased but due to the variability of  $91Y/lov^{def}$ , this decrease is not statistically significant. For  $lov^{10}$  and  $lov^{47}$ , there is a statistically significant decrease in the non-directed courtship. A one-way ANOVA of the hemizygous mutants was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the hemizygous mutant lines to the control line (Ore R/def). No statistical significant difference was found between the hemizygous males. A T-test was then used to compare the original line to the line over deficiency, which identified statistically significant differences for  $lov^{10}$ , and  $lov^{47}$ . ( $p=0.779$ ; Ore R and Canton S  $n=19$ ; OreR/def  $n=7$ ; 91Y  $n=10$ ; 91Y/def  $n=15$ ;  $lov^{10}$   $n=13$ ;  $lov^{10}/def$   $n=22$ ;  $lov^{38}$   $n=19$ ;  $lov^{38}/def$   $n=26$ ;  $lov^{47}$   $n=10$ ;  $lov^{47}/def$   $n=10$ ;  $lov^{65}$   $n=14$ ;  $lov^{65}/def$   $n=20$ ;  $lov^{66}$   $n=14$ ;  $lov^{66}/def$   $n=13$ )

two PCR products are produced, one 400 base pairs larger than the other. The smaller product is from the chromosome with the  $lov^{66}$  deletion. When the  $lov^{66}$  chromosome is placed over the  $lov^{def}$  chromosome, this PCR reaction should produce only the smaller fragment derived from the  $lov^{66}$  chromosome. However, if the  $lov^{def}$  does not remove the  $lov$  locus, two products will be produced. When this PCR reaction is performed on  $lov^{66}/lov^{def}$  flies, only the smaller band appears. Thus, the  $lov^{def}$  is still present on the chromosome.

One possibility then is that the courtship defects observed are not due to the mutations at the  $lov$  locus. However, as shown in Chapter 4, analysis of expression of  $lov$  in the PNS of  $lov$  mutants embryos suggest some  $lov$  mutants produce ectopic  $lov$  expression. Additionally, as shown in Chapter 8, over-expression of  $lov$  using the GAL4-UAS system results in altered courtship behavior. This suggests that it is the overexpression or ectopic expression of  $lov$  that is responsible for the altered courtship behavior. In the hemizygous condition, the level of  $lov$  expression in a mutant showing over-expression would be reduced to approximately wild type levels and the courtship behavior would be corrected. A second possible explanation of the improved Courtship Indices is that one of the other genes removed by the  $lov$  deficiency chromosome may also modify courtship behavior. By removing that gene, the courtship behavior is then corrected.

To further help assess whether the observed courtship defects were indeed due to the various  $lov$  mutations, a series of complementation crosses were performed and courtship assays were performed on the resulting offspring. If the observed courtship defects in each line are in fact due to a mutation in  $lov$ , the offspring from each of the



complementation crosses should retain some courtship defects. If the observed courtship defects are not due to a mutation at the *lov* locus, but rather to different secondary mutations elsewhere on the chromosome, it would be expected that there no longer would be courtship defects in these mutant combinations.

Some predictions as to what the courtship behavior in the various complementation crosses can be made based on the location of the deletions and the behaviors of the different lines in the courtship assays. In *lov*<sup>66</sup>, there is a 372 base pair deletion down of the 91Y insertion site. *lov*<sup>38</sup> and *lov*<sup>47</sup> have 600 base pair deletion and 1400 base base pair deletion upstream of the 91Y insertion site respectively. All the lines, with the exception of *lov*<sup>66</sup>, have a significantly lower Courtship Index than wild type. Therefore, it would be expected that when *lov*<sup>10</sup>, *lov*<sup>47</sup>, and *lov*<sup>65</sup> are crossed to *lov*<sup>38</sup> the resulting offspring would have low Courtship Indices similar to either parent line. In contrast, when *lov*<sup>66</sup> is crossed to either *lov*<sup>38</sup> or *lov*<sup>47</sup>, the resulting offspring would have a courtship index similar to *lov*<sup>66</sup> and the wild type control. This is because the deletions in *lov*<sup>66</sup> and *lov*<sup>38</sup> or *lov*<sup>47</sup> would compensate for one another. *lov*<sup>38</sup> was crossed to each of the other *lov* mutant lines. *lov*<sup>38</sup> was chosen because it had the lowest Courtship Index of all the mutant lines. *lov*<sup>47</sup> and *lov*<sup>66</sup> were also crossed since their respective deletions remove DNA on opposite sides of the insertion site and the *lov*<sup>47</sup> deletion is larger than the *lov*<sup>38</sup> deletion. Courtship assays were performed as described in Materials and Methods.

When the directed Courtship Index was calculated it was found that *lov*<sup>38</sup>/*lov*<sup>10</sup> has index of 0.12. *lov*<sup>38</sup>/*lov*<sup>47</sup> has a courtship index of 0.22. *lov*<sup>38</sup>/*lov*<sup>65</sup> has a courtship index of 0.21. All of these Courtship Indexes are statistically similar to both parent lines. In contrast *lov*<sup>38</sup>/*lov*<sup>66</sup> had a directed Courtship Index of 0.35 which is statistically

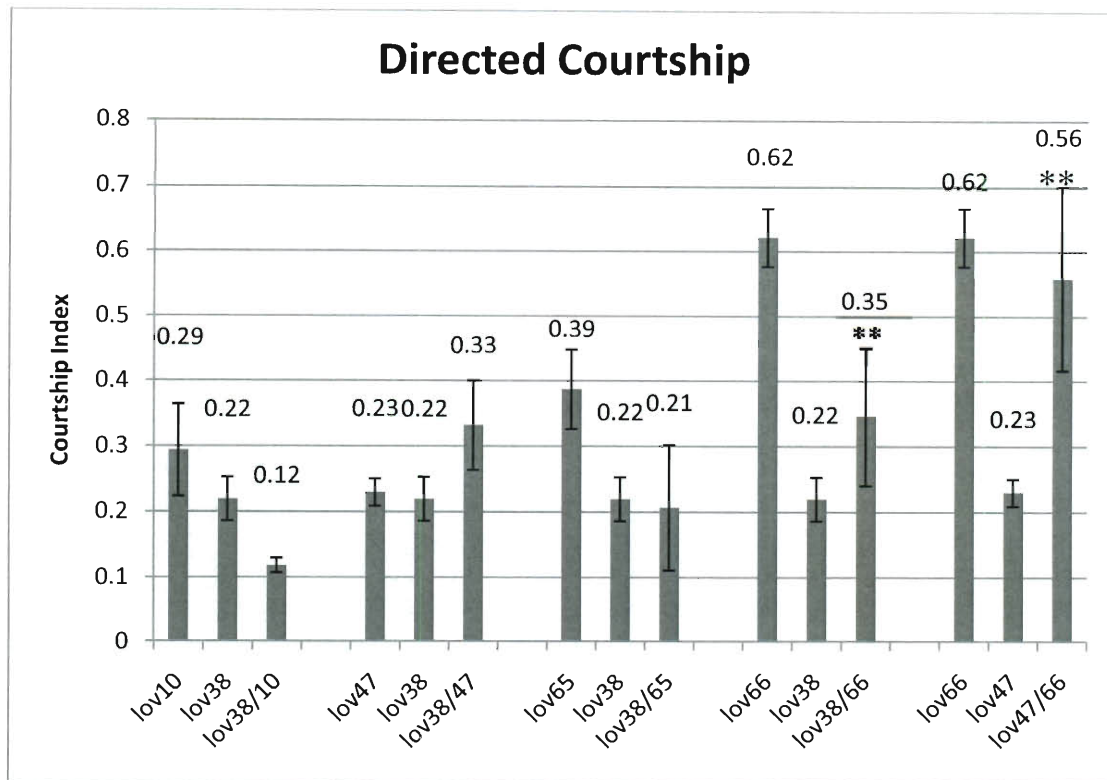


Figure 6-10: Directed courtship for complementation crosses between lov mutants.  $lov^{38}/lov^{10}$ ,  $lov^{38}/lov^{47}$ , and  $lov^{38}/lov^{65}$  had similar CI as either parent line.  $lov^{38}/lov^{66}$  had a directed CI that was statistically significantly lower compared to  $lov^{66}$ .  $lov^{47}/lov^{66}$  had a directed CI that is similar to the directed Courtship Index of  $lov^{66}$  and statistically significantly higher compared to  $lov^{47}$ . A one-way ANOVA was used to compared each combination of lov mutant alleles with both of its parent lines. If the differences in the CIs were found to be statistically significant, a T-test was used to compare each combination of lov mutant alleles against each of its parent lines. ( $lov^{10}/lov^{38}$ :  $p = 0.171$ ;  $lov^{10} n = 13$ ;  $lov^{38} n = 19$ ;  $lov^{38}/lov^{10} n = 8$ ;  $lov^{47}/lov^{38}$ :  $p = 0.332$ ;  $lov^{47} n = 10$   $lov^{38}/lov^{47} n = 9$ ;  $lov^{65}/lov^{38}$ :  $p = 0.095$ ;  $lov^{65} n = 14$ ;  $lov^{38}/lov^{65} n = 10$ ;  $lov^{66}/lov^{38}$ :  $p \leq 0.001$ ;  $lov^{66} n = 14$ ;  $lov^{38}/lov^{66} n = 10$ ;  $lov^{66}/lov^{47}$ :  $p = 0.0004$ ;  $lov^{47} n = 10$ ;  $lov^{66} n = 14$ ;  $lov^{47}/lov^{66} n = 8$ )

significantly lower than  $lov^{66}$ . For  $lov^{47}/lov^{66}$ , the directed Courtship Index is 0.56 which is similar to the directed Courtship Index of  $lov^{66}$  (Figure 6-10).

When the non-directed Courtship Index was calculated, it was found only  $lov^{38}/lov^{10}$  had a statistically significant higher non-directed courtship compared to  $lov^{38}$ .  $lov^{38}/lov^{47}$ ,  $lov^{38}/lov^{65}$ , and  $lov^{38}/lov^{66}$  all had non-directed Courtship Indexes that were not statistically significant from either parent line. Finally,  $lov^{47}/lov^{66}$  had a non-directed Courtship Index that is statistically significant lower compared to  $lov^{47}$  (Figure 6-11).

The results largely fit with predictions based on the location of the mutations. For each of the lines crossed to  $lov^{38}$ , with the exception of  $lov^{66}$ , the Courtship Index for the resulting offspring remains decreased compared to wild type. The offspring from  $lov^{66}$  crossed to either  $lov^{38}$  or  $lov^{47}$  had a Courtship Index that was higher compared to  $lov^{38}$  or  $lov^{47}$  parent line.

With the exception of  $lov^{66}$ , it appears that the various mutation in *lov* cause a decrease in the courting of females. Male-female courtship may not be the only courtship behavior affected by the mutations. Given the similarities between *lov* and *fru* and that fact that mutations in *fru* cause a male-male courtship phenotype, it is possible that various *lov* mutants court both males and females. To test for this possibility, a male-male courtship assay was performed.

In male-male courtship assay, naïve males were collected and allowed to age seven days in individual vials. They were then placed in courtship wheels with naïve males control males which had also been allowed to age for seven days in individual vials and recorded for 10 minutes (O'Dell, 2003). The amount of time that test male spent actively courting the target male was recorded and the Courtship Index was determined.

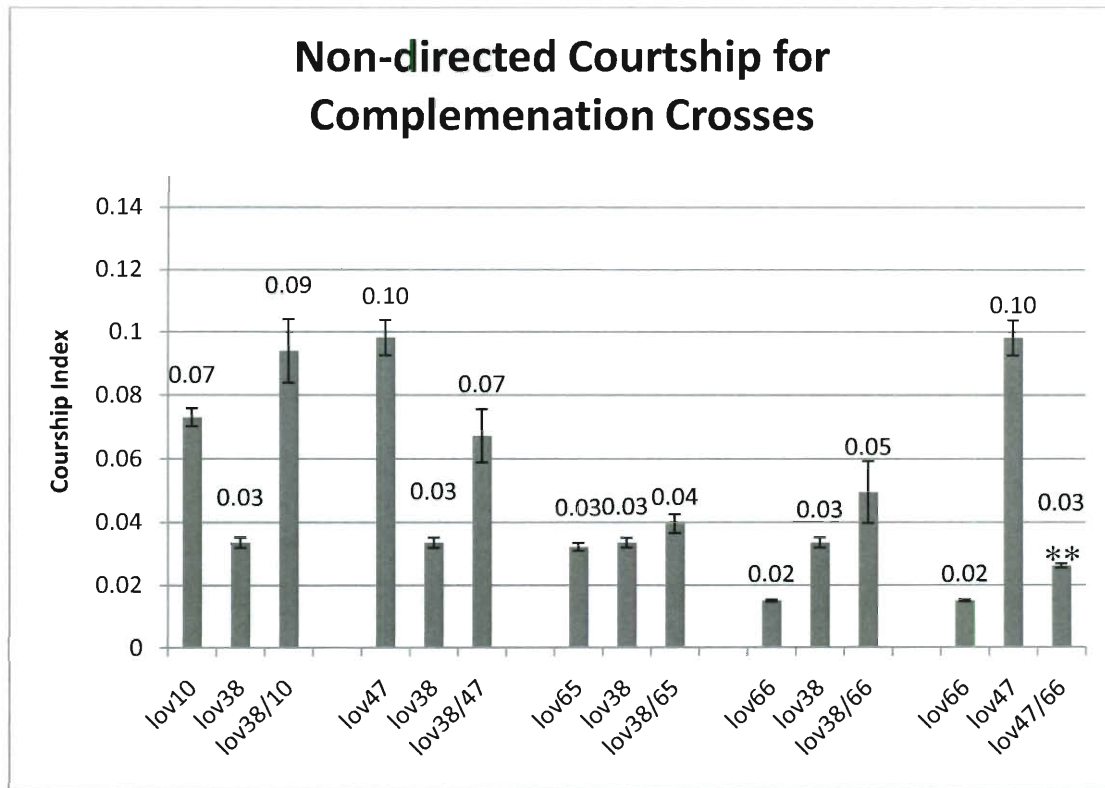


Figure 6-11: Non-directed courtship for complementation crosses of lov mutants.

When the non-directed CI was calculated, it was found only  $\text{lov}^{38}/\text{lov}^{10}$  had a statistically significant increase in non-directed courtship compared to  $\text{lov}^{38}$ .  $\text{lov}^{38}/\text{lov}^{47}$ ,  $\text{lov}^{38}/\text{lov}^{65}$ , and  $\text{lov}^{38}/\text{lov}^{66}$  all had non-directed CIs that were not statistically significant from either parent line.  $\text{lov}^{47}/\text{lov}^{66}$  had a non-directed CI that is statistically significant lower compared to  $\text{lov}^{47}$ . A one-way ANOVA was used to compare each combination of lov mutant alleles with both of its parent lines. If the differences in the CIs were found to be statistically significant, a T-test was used to compare each combination of lov mutant alleles against each of its parent lines. ( $\text{lov}^{10}/\text{lov}^{38}$ :  $p=0.045$ ;  $\text{lov}^{10}$   $n=13$ ;  $\text{lov}^{38}$   $n=19$ ;  $\text{lov}^{38}/\text{lov}^{10}$   $n=8$ ;  $\text{lov}^{47}/\text{lov}^{38}$ :  $p=0.043$ ;  $\text{lov}^{47}$   $n=10$ ;  $\text{lov}^{38}/\text{lov}^{47}$   $n=9$ ;  $\text{lov}^{65}/\text{lov}^{38}$ :  $p=0.899$ ;  $\text{lov}^{65}$   $n=14$ ;  $\text{lov}^{38}/\text{lov}^{65}$   $n=10$ ;  $\text{lov}^{66}/\text{lov}^{38}$ :  $p=0.320$ ;  $\text{lov}^{66}$   $n=14$ ;  $\text{lov}^{38}/\text{lov}^{66}$   $n=10$ ;  $\text{lov}^{66}/\text{lov}^{47}$ :  $p=0.0002$ ;  $\text{lov}^{47}/\text{lov}^{66}$   $n=8$ )

As discussed earlier, all males isolated for seven days show some indiscriminate courtship. This phenomenon is reflected in the Courtship Index of the control males. As shown in Chapter 5, *lov*<sup>66</sup> males court other males and have a male-male Courtship Index of 0.13 (Figure 6-12). For all the lines, the male-male Courtship Index is similar to that of control males (Figure 6-12). This result is interesting as the location of deletion in *lov*<sup>66</sup> is in the same location as the one of the small deletions in *lov*<sup>10</sup> and *lov*<sup>65</sup>. This suggests the possibility that *lov* regulates different aspects of courtship through the uses of different enhancers and the *lov*<sup>66</sup> disrupts one enhancer or set of enhancers and the other mutations disrupts a different enhancer or set of enhancers. The set of enhancers that are disrupted by *lov*<sup>66</sup> may not be disrupted in *lov*<sup>10</sup> and *lov*<sup>65</sup>, accounting for the difference in behavior between the lines.

To further understand how *lov* is functioning in normal courtship, a modified male-male courtship assay was performed. In these experiments, the *lov* mutant males were placed in a courtship chamber paired with another male that appears to be male, both in behavior and appearance. However, he has a female pheromone profile. This is accomplished through the use of the GAL4-UAS system where in the males selectively express Tra (a sex specific protein normally only expressed in females) in oenocytes. This selective expression results in males expressing a female pheromone profile (Bray and Amrein, 2003). If the *lov* mutant defects in courting females are due to a defect in detecting or processing the female pheromones, they should show no courtship of the feminized males. If their defects have other origins, they will show an increased response compared to the male-male courtship.

As can be seen from Figure 6-13, all the lines have an increase in Courtship

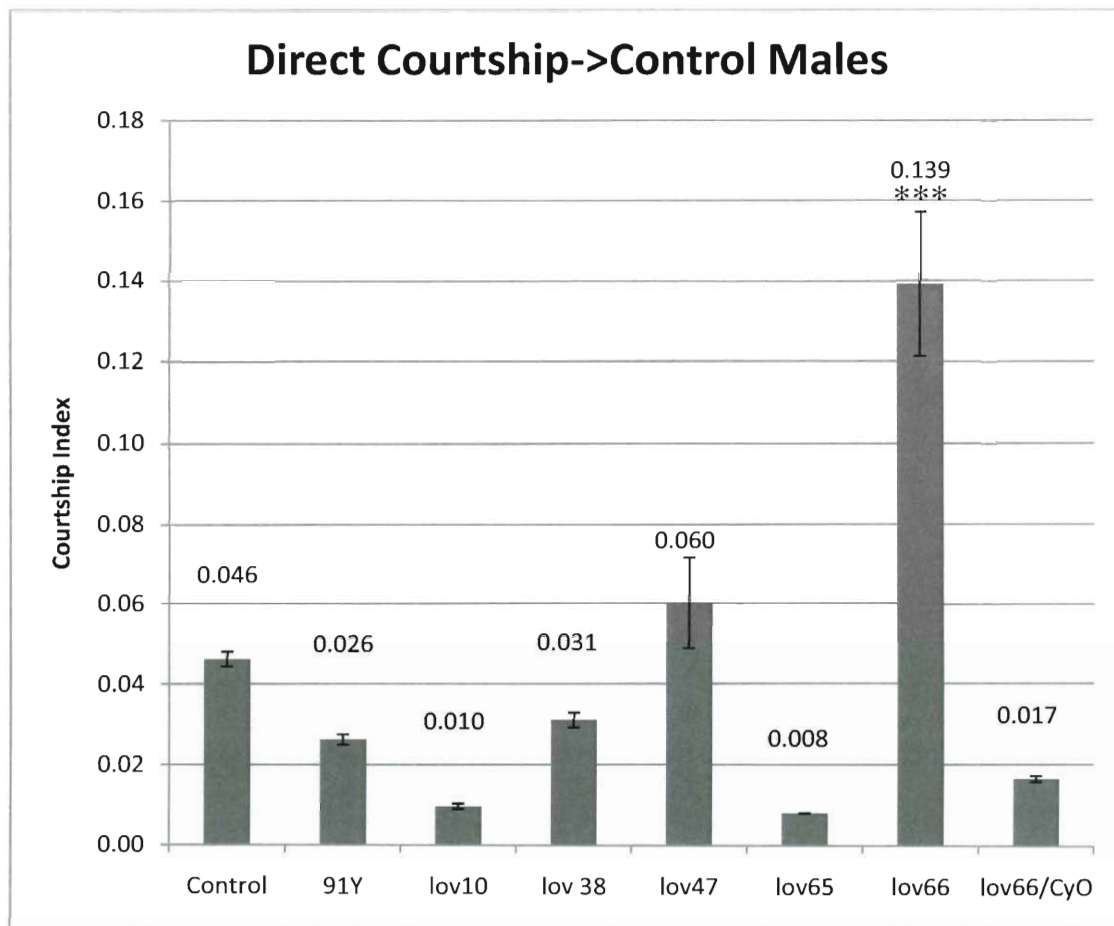


Figure 6-12: Directed Courtship Towards Control Males. For all the lines, with the exception of lov<sup>66</sup>, the Courtship Index is similar to that of control males. However, the Courtship Index for lov<sup>66</sup> males is higher than the control line. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line, Ore R. (p=0.0003; Ore R n=10; 91Y n=10; lov<sup>10</sup> n= 10; lov<sup>38</sup> n= 10; lov<sup>47</sup> n= 10; lov<sup>65</sup> n= 10; lov<sup>66</sup> n=13; lov<sup>66</sup>/CyO n=10)

Index in the modified male-male courtship assay as compared to the original male-male courtship assay. Additionally,  $lov^{38}$ ,  $lov^{66}$ , and  $lov^{66}/CyO$  have similar Courtship Indexes as the control males. For  $lov^{66}$  and  $lov^{66}/CyO$ , this is not a surprising result. For 91Y,  $lov^{10}$ ,  $lov^{47}$ , and  $lov^{65}$ , the Courtship Index is about half of that of the control Courtship Index, but this decrease is not statistically significant. However, the behavior of 91Y,  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{47}$ , and  $lov^{65}$  males in the assay was very different compared to control or  $lov^{66}$  and  $lov^{66}/CyO$  males. As when partnered with females, these males made very few attempts at courting the feminized male, typically only when the feminized males were in close proximity. The courting encounters (were the male is actively courting the feminized male) only lasted a few seconds on average and were about half the amount of time as the control males. Further, the control,  $lov^{66}$  and  $lov^{66}/CyO$  males all made attempts to copulate the feminized males, but the other  $lov$  mutant males did not.

When non-directed courtship was examined, the non-directed Courtship Index increased for 91Y,  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{47}$ , and  $lov^{65}$  as compared to male-female courtship (Figure 6-14). There was little to no non-directed courtship for  $lov^{66}$  and  $lov^{66}/CyO$  males, which is similar to male-female courtship.

The wild type directed courtship levels are reduced in this assay compared to courtship with females. This confirms that other cues, such as appearance, are important in courtship. All of  $lov$  mutants have directed courtship levels that are not significantly different from wild type.  $lov^{66}$  and  $lov^{66}/CyO$  also behaved similarly in the courtship assay ( similar length of courtship encounters and little non-directed courtship), suggesting that their ability to detect and process female pheromones is similar to that of wild type. In contrast, 91Y,  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{47}$ , and  $lov^{65}$  all a higher level of non-directed

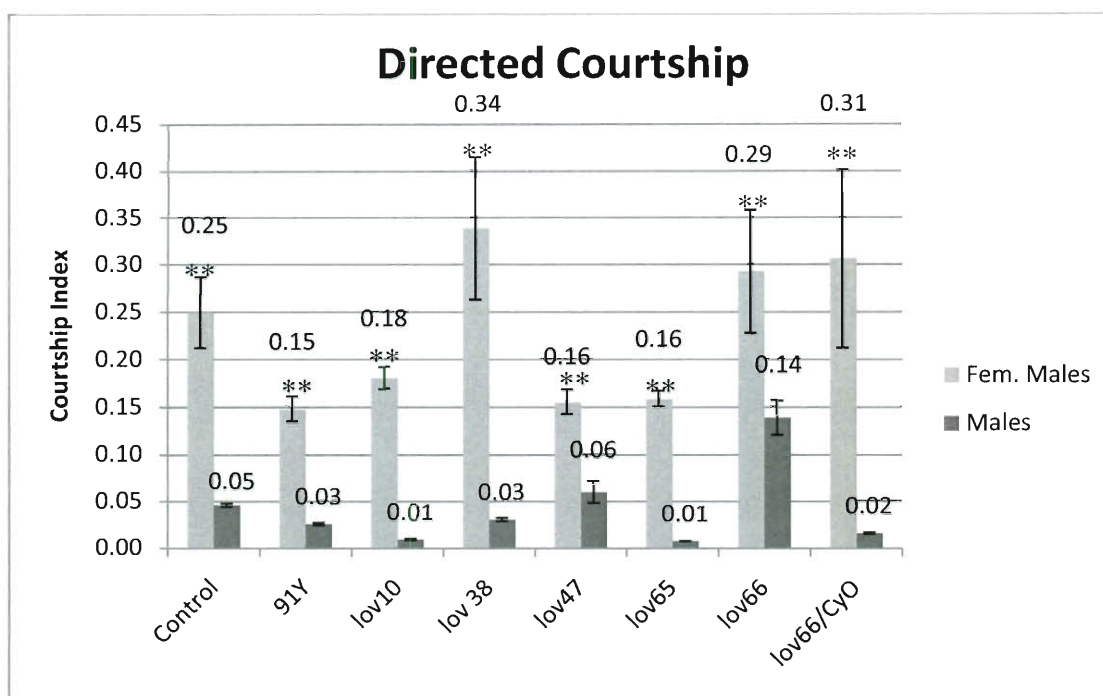


Figure 6-13: Comparison of directed courtship for the *lov* mutants pursuing males or feminized males. All the lines have an increase in CI in the modified male-male courtship as compared to the original male-male courtship. *lov*<sup>38</sup>, *lov*<sup>66</sup>, and *lov*<sup>66</sup>/CyO have similar CIs as the control males. For 91Y, *lov*<sup>10</sup>, *lov*<sup>47</sup>, and *lov*<sup>65</sup>, the feminized CI is about half of that of the control feminized CI. However, the difference in the CIs is not statistically significant. A one-way ANOVA was performed to determine if there were differences among the lines when paired with a feminized male. A Dunnett's Test was then used to compare the results of the mutant lines to the control line, Ore R when paired with a feminized male. No statistical significant difference was found. A T-test was performed for each line between the test male pair with feminized male and a wild type male. Every line had a higher directed CI with the feminized male that was statistically significant compare to wild type males. ( $p=0.187$ ; Ore R  $n=14$ ; 91Y  $n=10$ ; *lov*<sup>10</sup>  $n=10$ ; *lov*<sup>38</sup>  $n=9$ ; *lov*<sup>47</sup>  $n=10$ ; *lov*<sup>65</sup>  $n=10$ ; *lov*<sup>66</sup>  $n=13$ ; *lov*<sup>66</sup>/CyO  $n=10$ )



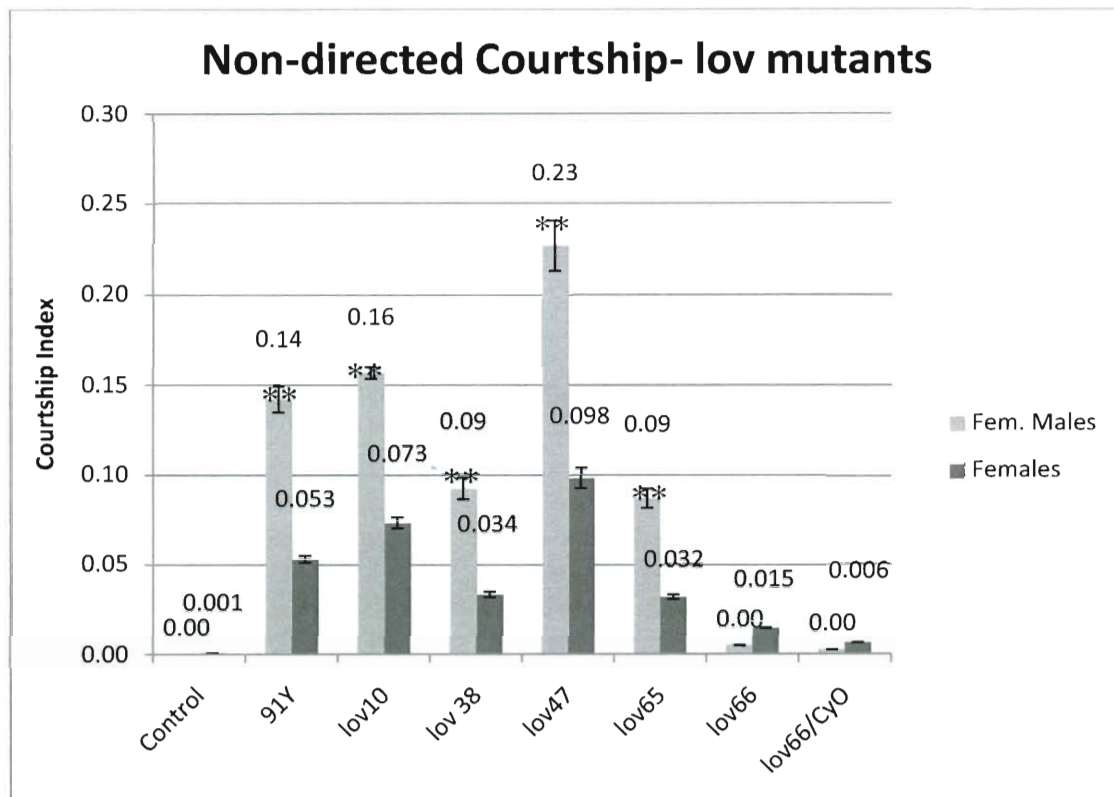


Figure 6-14: Comparison of non-directed courtship for the lov mutants pursuing females or feminized males. The non-directed Courtship Index increased for 91Y, lov<sup>10</sup>, lov<sup>38</sup>, lov<sup>47</sup>, and lov<sup>65</sup> as compared to male-female courtship. There was little to no non-directed courtship for the control, lov<sup>66</sup> and lov<sup>66</sup>/CyO males. A one-way ANOVA was performed to determine if there were differences among the lines when paired with a feminized male. A Dunnett's Test was then used to compare the results of the mutant lines to the control line, Ore R when paired with a female. No statistical significant difference was found. A T-test was performed for each line between the test male pair with feminized male and a wild type female. Every mutant line, except lov<sup>66</sup> and lov<sup>66</sup>/CyO, had a higher directed CI with the feminized male that was statistically significant higher compared to females. ( $p \leq 0.001$ ; Ore R n=14; 91Y n=10; lov<sup>10</sup> n= 10; lov<sup>38</sup> n= 9; lov<sup>47</sup> n= 10; lov<sup>65</sup> n= 10; lov<sup>66</sup> n=13; lov<sup>66</sup>/CyO n=10)

courtship compared to the control when paired either with a female or feminized male and shorter courtship encounters. This would suggest that these mutants are able to detect female pheromones but may have difficulties locating and/or processing them.

### Summary

The above courtship assays demonstrate that *lov* has a role in normal courtship. Ectopic expression of *lov* appears to lead to a reduction in courtship. Additionally, mutations in *lov* cause alterations in courtship such as attempts at copulation in wrong locations, passive courtship, and non-directed courtship. Mutations in *lov* also cause males to court other males. Finally, based on the modified male-male courtship assay, mutations in *lov* may affect a male's ability to locate and/or process female pheromones. This suggests that *lov* functions to regulate normal courtship through the identification of correct courtship objects as well as the regulation of the various courtship steps. How *lov* functions to regulate courtship is not clear from these courtship assays. *lov* has been shown to be expressed in both the CNS and PNS. Therefore, it is possible that *lov* is regulating courtship by sensory input in the PNS or in processing in the CNS. The modified male-male courtship assays show *lov* may be involved in olfactory input and that *lov* may function in the PNS to regulate courtship behavior.

## Materials and Methods

### Courtship Assays

Naïve males were collected and allowed to age seven days in individual vials. They were then placed in courtship wheels with a partner, either 3-5 day old Control females (Ore R or Canton S) virgin females or 7-day old males ( $w^1$ ; Sco/CyO for male-male assays or feminized males) aged in individual vials. Each pair was recorded for 10 minutes and the amount of time a male courted its partner was determined (O'Dell, 2003). That time was then used to calculate the Courtship Index (CI), which is the percentage of the time the male spends courting the female (Villella et al, 1997).

### Statistical Analysis

For each courtship assay, a one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control line. A t-test was performed to determine if there is a difference for a line under different conditions. The test used for each set of experiments is stated in the figure for each experiment. An alpha of 0.05 was used for all statistics. The number of males (n) tested and the p-value is listed in each figure legend. Statistical significance is indicated in each figure in the following way: \* =  $p \leq 0.05$ ; \*\* =  $p \leq 0.01$ ; \*\*\* =  $p \leq 0.001$ .

## CHAPTER 7: OTHER BEHAVIORS AFFECTED BY THE *lov* MUTATIONS

What is responsible for the observed courtship defects in the various *lov* mutant lines?

Previous work and my own studies have shown that *lov* is specifically expressed in both the PNS and the CNS. This suggests that the defects in courtship could be due to defects in sensory input in the PNS or to processing in the CNS. The highest predicted level of *lov* expression is in the head (Flyatlas.org), suggesting the possibility that *lov* is acting in the CNS to mediate courtship behavior. However, data collected in our lab (see Chapter 4) and in others suggest the *lov* maybe functioning in the PNS through sensory inputs (gustatory, olfactory, vision) and motility to mediate courtship behavior. To determine if this is in fact the case, a series of behavioral assay were performed.

Does a defect in gustatory response contribute to the observed courtship defects?

EYFP expression in 91Y adult legs has shown expression of *lov* in the legs, particularly in the tarsus, which is important for taste. Given this expression pattern, it is possible that the courtship defects observed could be due in part to a failure of males to properly taste and/or respond to the gustatory input of the females. Furthermore, other mutations which disrupt male specific taste receptors in the front legs result in males that only perform the first two steps of courtship—orientation and tapping—and which fail to copulate (Bray and Amrein, 2003). It is believed that these mutants can not determine if the females are appropriate courtship objects due to their inability to taste. Therefore, given the expression pattern of *lov* in the tarsus, it is possible that *lov* may be involved in sensory input in the form of tasting and that in the mutants the gustatory response to the females is altered.

To test for this possibility, the Proboscis Extension Response (PER) assay

previously described by Deak (1976) was performed (see materials and methods). Different gustatory receptors in males detect sweet stimuli, bitter stimuli, and female pheromones. The PER was used to determine if the general gustatory response is intact in the various *lov* mutant lines. If the various *lov* mutations affect the flies' abilities to taste because they disrupt the function of a specific class of gustatory neurons, the response to a single tastant should be disrupted and responses to the other tastants should be normal. However, if the different mutants affect all the gustatory receptors neurons or the central processing of the gustatory input, then the response to all tastants would be abnormal. The basis for the PER assay is that when an attractive (typically sweet) tastant, such as glucose or sucrose, is touched to the taste receptors in the front leg, a fly will extend its proboscis (Figure 7-1). When a repellent (bitter) tastant, such as caffeine, is touched to the front leg, the fly does not extend its proboscis even in the presence of an attractive tastant. The assay was used to determine whether or not the various *lov* mutants can taste properly.

The lines tested with this assay were Ore R (wild type control), 91Y (original *lov* mutant), *lov*<sup>10</sup>, *lov*<sup>65</sup>, *lov*<sup>38</sup>, *lov*<sup>47</sup>, *lov*<sup>66</sup>, and *lov*<sup>66</sup>/CyO. As can be seen from Figure 7-2, for all the lines tested except *lov*<sup>66</sup>, and *lov*<sup>66</sup>/CyO, at least 70% of the flies extended for both 4% sucrose and 1% sucrose. This suggests for all the lines other than *lov*<sup>66</sup>, and *lov*<sup>66</sup>/CyO, the flies are able to detect and respond properly to an attractive (sweet) tastant.

For all eight lines, at least 75% of flies tested did not extend for the 4% caffeine+4% sucrose, suggesting that all the lines are able to detect and respond properly

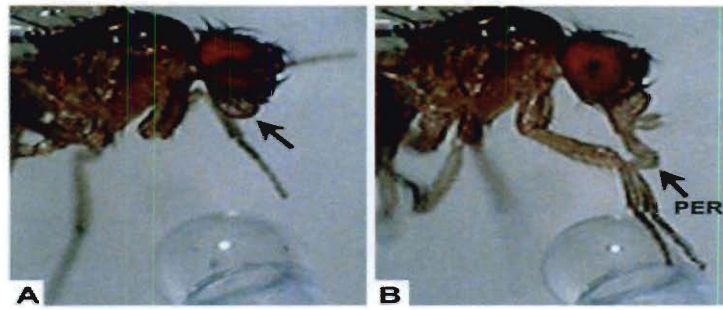


Figure 7-1: Proboscis Extension Response (PER) Assay. In this assay, either an attractive tastant or a mixture of an attractive tastant and repellent tastant are touched to the front leg of a fly (A). If the fly can taste and respond properly, the fly will extend its proboscis in response to the attractive tastant (B). It will not extend its proboscis to the attractive-repellent tastant mix. Modified from Gordesky-Gold, B. et al., (2008).

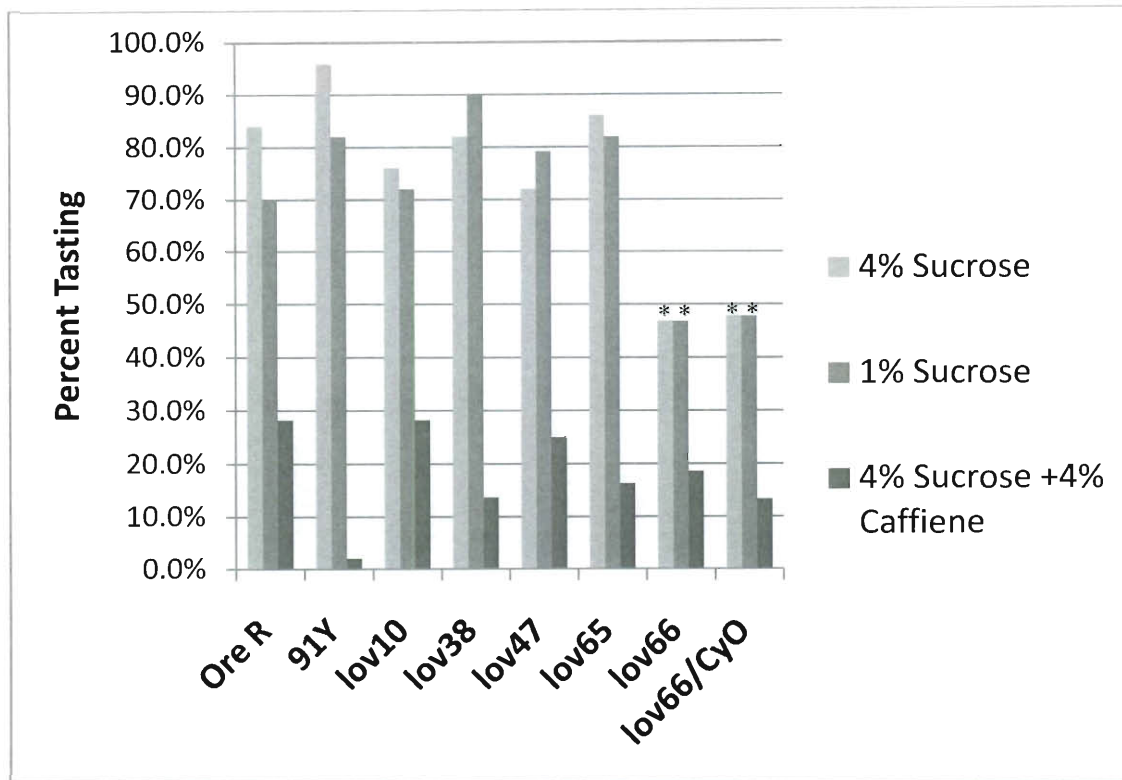


Figure 7-2: Taste testing of the various lov mutants. For all but lov<sup>66</sup> and lov<sup>66</sup>/CyO, at least 70% of the flies extended for both the 4% and the 1% sucrose solutions. This suggest that all the lines other than lov<sup>66</sup> and lov<sup>66</sup>/CyO can taste and respond to an attractive tastant. It is possible that lov<sup>66</sup> and lov<sup>66</sup>/CyO have defects in taste and/or formulating the correct response to tastant. In contrast, less than 25% of the flies in each line extended for the sucrose plus caffeine solution, suggesting that all lines can taste and respond to a repellant tastant properly. (4% Sucrose: Ore R n=50, 91Y n=50, lov<sup>10</sup> n=50; lov<sup>38</sup> n=50, lov<sup>47</sup> n=50, lov<sup>65</sup> n=50, lov<sup>66</sup> n=47, lov<sup>66</sup>/CyO n=44;  $\chi^2_{CS, 91Y}=5.36$ ;  $\chi^2_{CS, lov10}=2.38$ ;  $\chi^2_{CS, lov38}=0.150$ ;  $\chi^2_{CS, lov47}=5.36$ ;  $\chi^2_{CS, lov65}=0.150$ ;  $\chi^2_{CS, lov66}=48.4$ ;  $\chi^2_{CS, lov66/CyO}=43.1$ ; 1% Sucrose: Ore R n=50, 91Y n=50, lov<sup>10</sup> n=50; lov<sup>38</sup> n=50, lov<sup>47</sup> n=48, lov<sup>65</sup> n=50, lov<sup>66</sup> n=47, lov<sup>66</sup>/CyO n=44;  $\chi^2_{CS, 91Y}=3.43$ ;  $\chi^2_{CS, lov10}=0.100$ ;  $\chi^2_{CS, lov38}=9.52$ ;  $\chi^2_{CS, lov47}=1.92$ ;  $\chi^2_{CS, lov65}=3.43$ ;  $\chi^2_{CS, lov66}=12.0$ ;  $\chi^2_{CS, lov66/CyO}=10.4$ ;

(Figure7-2 cont). 4% Sucrose and 4% Caffeine: Ore R n=50, 91Y n=50, lov<sup>10</sup> n=50;  
 lov<sup>38</sup> n=50, lov<sup>47</sup> n=50, lov<sup>65</sup> n=50, lov<sup>66</sup> n=51, lov<sup>66</sup>/CyO n=51;  $\chi^2_{CS, 91Y}=3.84$ ;  $\chi^2_{CS, lov10}=0.00$ ;  $\chi^2_{CS, lov38}=2.91$ ;  $\chi^2_{CS, lov47}=0.120$ ;  $\chi^2_{CS, lov65}=1.86$ ;  $\chi^2_{CS, lov66}=1.18$ ;  $\chi^2_{CS, lov66/CyO}=3.11$ )



to a repellent (bitter) tastant (Figure 7-2). Therefore, with the exception of  $lov^{66}$ , and  $lov^{66}/CyO$ , the various  $lov$  mutants can taste and respond properly to both attractive and repellent tastants. This suggests that these lines (91Y,  $lov^{10}$ ,  $lov^{65}$ ,  $lov^{38}$ ,  $lov^{47}$ ) can taste and respond properly and that the courtship defects are likely not due to the males inability to taste the female.  $lov^{66}$  and  $lov^{66}/CyO$  appear to be able to detect and respond to repellent tastants properly but not to attractive tastants. This suggests a defect in proper tasting ability and may be what is partially responsible for the observed courtship defects. Given the embryonic expression of  $lov$ , is the gustatory response in larvae altered in the  $lov$  mutants?

In addition to examining the various lines' ability to taste in adults, we also examined the larval taste ability. We have previously shown widespread expression of  $lov$  in the embryonic PNS. As the embryonic nervous system sets up the pattern for the larval nervous system, experiments were performed to determine what, if any effect the mutant alleles of  $lov$  would have on the larval tasting ability using a larval taste assay (Figure 7-3) (see material and methods). As with the adults, the larvae were exposed to both an attractant tastant (4% sucrose) and a repellent tastant (4% caffeine+ 4% sucrose) and the responses were observed.

The lines tested with this assay were Ore R (wild type control), 91Y (original  $lov$  mutant),  $lov^{10}$ ,  $lov^{65}$ ,  $lov^{38}$ , and  $lov^{47}$ . The lines  $lov^{66}$  and  $lov^{66}/CyO$  were not tested due to an inability to distinguish between the homozygous and heterozygous larvae. Results show that for all the lines tested, the larvae responded appropriately (Figure 7-4). For sucrose experimental plates, the scores were positive, meaning that the larvae could taste and respond appropriately to sucrose. There was no statistically significant difference

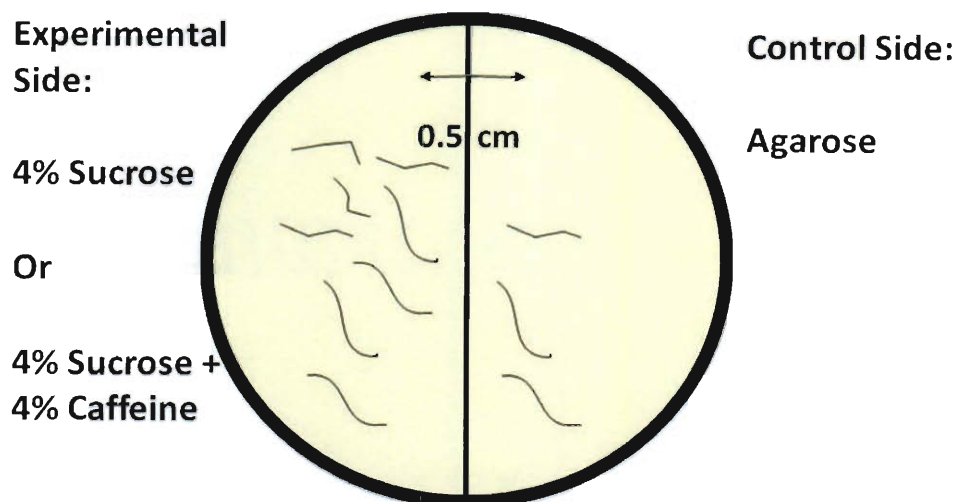


Figure 7-3: Larval Taste Test. For this experiment, A large petri dish was divided in half with one half containing only agarose and the other half containing agarose and 4% sucrose (attractant plate) or 4% sucrose and 4% caffeine (repellant plate). If the larvae on the attractant plate can taste and respond appropriately, the majority of larvae should be on the side that contains the sucrose. If the larvae on the repellant plate can taste and respond appropriately, the majority of larvae should be on the side that contains only agarose. To score this assay, the number of larvae that were more than 0.5 cm from the junction of control (plain) agarose and experimental (sucrose or sucrose plus caffeine) agarose were counted. The number on the control side (agarose) was subtracted from the number on the experimental side (sucrose or sucrose plus caffeine) and the resulting number was divided by the total number of larvae to provide the normalized score, which is the larval response index (LRI). If on the sucrose plates, the LRI was positive, it means that the larvae can taste and respond appropriately to sucrose. If the LRI is negative on the sucrose plate, it means that the larvae cannot taste and/or respond appropriately to sucrose. If the LRI is negative on the sucrose plus caffeine plate, it either means that the larvae cannot taste and/or respond appropriately to caffeine or are attracted to caffeine when they should be repelled by it.

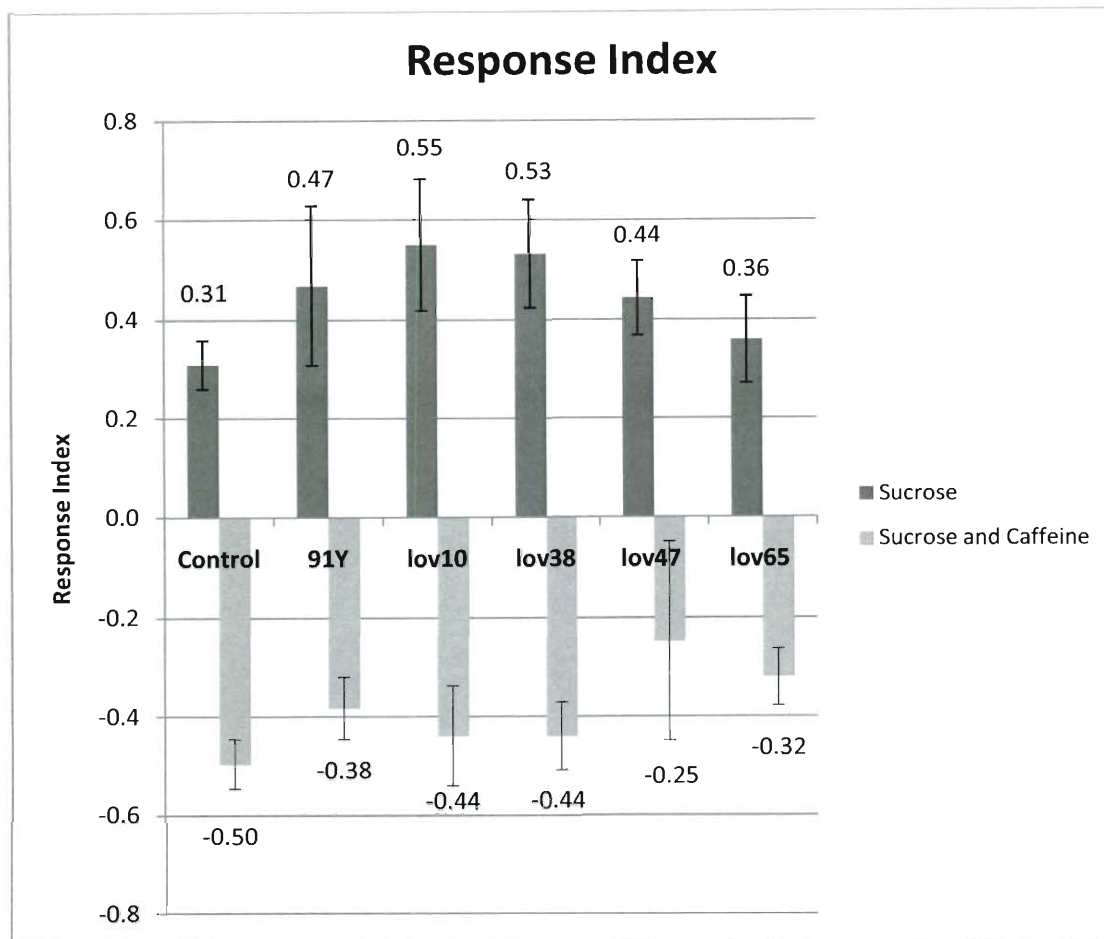


Figure 7-4: Larval taste testing for the various lov mutants. For all the lines, the larvae were attracted to the sucrose on the sucrose/agarose plates and avoided the sucrose plus caffeine on the sucrose plus caffeine/agarose plate, suggesting that the larvae from all three lines can taste and respond properly. There was no statistically significant difference between the control line and the lov mutants. A one-way ANOVA was performed for both condition to determine if there was a statistically significant difference between the lines.(4% Sucrose:  $p = 0.465$ ; Control=Ore R  $n=253$ , 91Y  $n=253$ , lov<sup>10</sup>  $n=253$ ; lov<sup>38</sup>  $n=260$ , lov<sup>47</sup>  $n=255$ , lov<sup>65</sup>  $n=256$ ; 4% Sucrose and 4% Caffeine:  $p = 0.468$ ; Control=Ore R  $n=249$ , 91Y  $n=304$ , lov<sup>10</sup>  $n=270$ ; lov<sup>38</sup>  $n=251$ , lov<sup>47</sup>  $n=253$ , lov<sup>65</sup>  $n=246$ )

between any of the lines to sucrose. For the caffeine plus sucrose plates, the LRI were negative, meaning the majority of the larvae avoided the side with caffeine, thus demonstrating that they could taste and respond to caffeine. Again, there was no statistically significant difference between the lines.

Does a defect in olfactory response contribute to the observed courtship defects?

Another sensory input that may be disrupted, thus affecting courtship in the various *lov* mutants is the smell. Anholt and Mackay (2001) identified a region of the second chromosome, which contains the *lov* locus, as important for olfaction. Because olfactory is important in *Drosophila* courtship, the ability of the *lov* mutants to smell was tested. A modified smell assay described by Anholt and Mackay was used. The lines tested in this assay were Canton S and Ore R (wild type controls), 91Y (original *lov* mutant), *lov*<sup>10</sup>, *lov*<sup>65</sup>, *lov*<sup>38</sup>, *lov*<sup>47</sup>, *lov*<sup>66</sup>, and *lov*<sup>66</sup>/CyO.

First, the response to water, a neutral odorant, was tested. If olfactory response of a particular line is intact, an ORI between two and three should be observed. As can be seen from Figure 7-6, the control line tested, Canton S, is slightly attracted to water (ORI=1.8). *lov*<sup>47</sup> appears to be slightly repelled by water (ORI=3.1). The remaining lines appear to be neutral to water, as would be expected.

Next, the ability of the lines to detect a repellent scent was tested. This was performed to determine whether gross olfactory function is intact in each fly line. For this assay, solutions of 1% benzaldehyde and 0.1% benzaldehyde were used as a repelling scent. Concentrations below 0.1% yielded a neutral response in the control flies, Ore R, thus suggesting that 0.1% benzaldehyde is the lowest concentration that flies can detect as repellent. For all the lines except *lov*<sup>47</sup>, the ORI for both 1% benzaldehyde and 0.1%

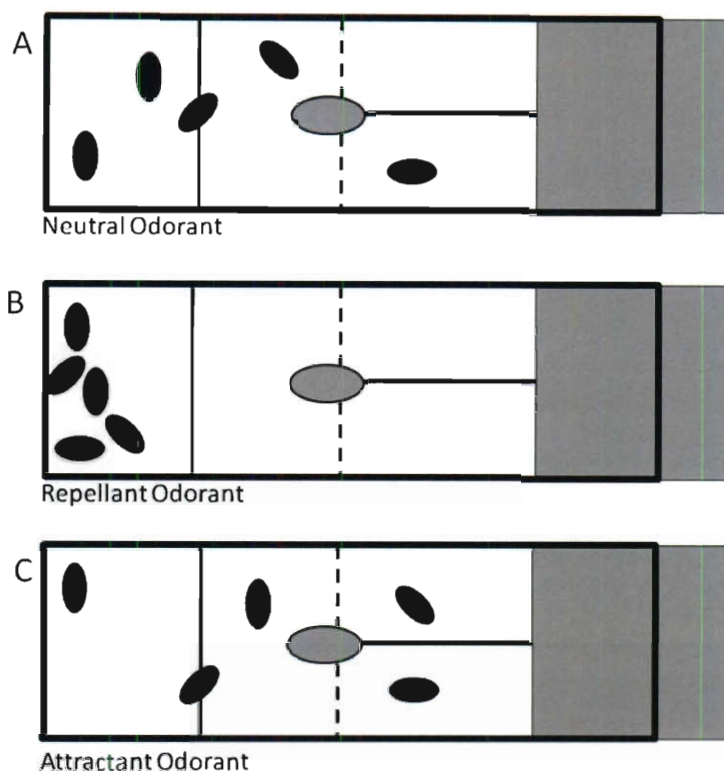


Figure 7-5: Smell Assay. A Q-tip soaked with 100  $\mu$ l of neutral, repellant, or attractive odorant was placed in a vial containing 5 4-6 day old males so that the tip of it was in the middle of the vial (dashed line). For one minute, the number of flies at the far end of the vial (opposite the plug, to the left of the solid line) was counted every five seconds. The number of flies at the far end was averaged to give an Olfactory Response Index (ORI). A. If the flies are evenly distributed throughout the vial, the line is neutral to the odorant and has an ORI between two and three B. If most of the flies ( $>3$ ) were at the far end of the vial (to the left of the dashed line), it means that line is repelled by the odorant and has an ORI above three. C. If most of the flies were at in the half of the vial containing the odorant near the plug, to the right of the dashed line) ( $>3$ ) it means that line is attracted to the odorant and the line has an ORI less than two.

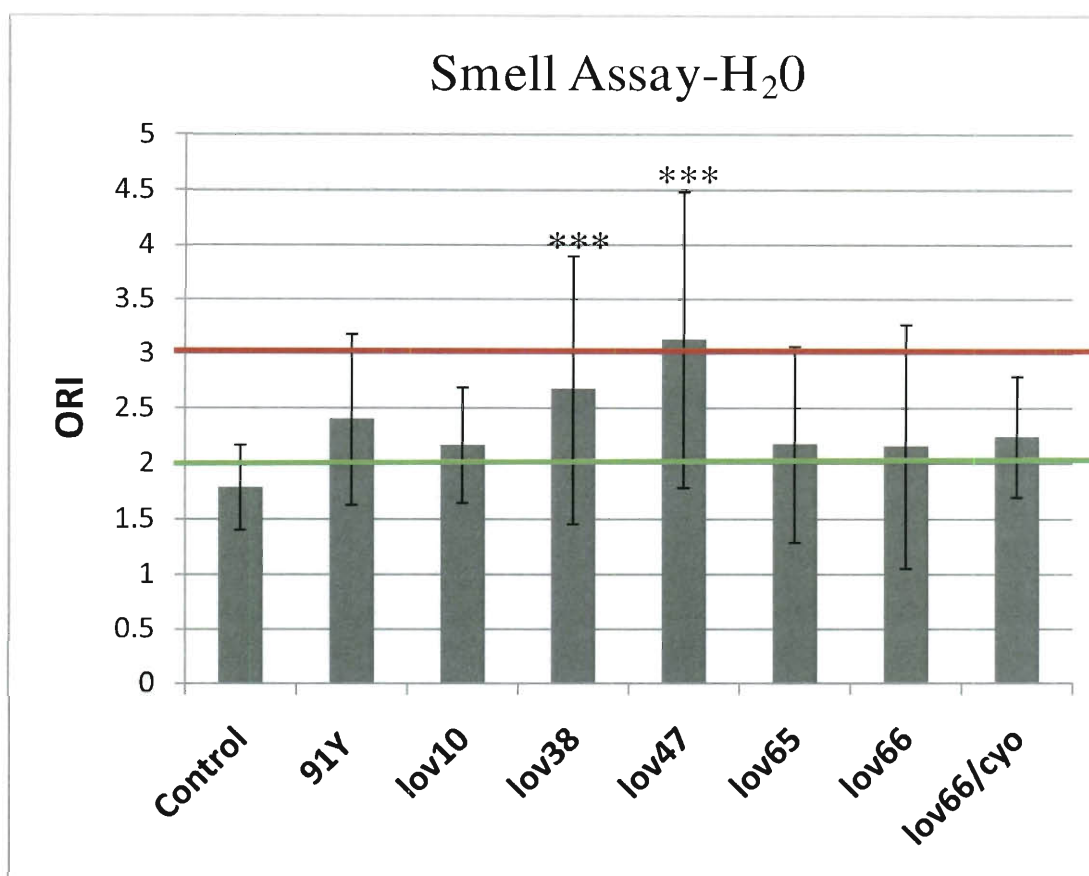


Figure 7-6: Olfactory Response Index (ORI) to water (neutral odorant) for the various lov mutants. An ORI between 2 and 3 (between the green and red lines) means flies are neutral to the odorant. For all the lines except Canton S (control) and lov<sup>47</sup>, the ORI was between 2 and 3, suggesting that all these lines can smell and respond properly. For Canton S, the ORI as 1.8 suggesting that this line is slight attracted to water. lov<sup>47</sup> has an ORI of 3.1 suggesting that this line is slightly repelled by water. Both lov<sup>38</sup> and lov<sup>47</sup> have ORI that are statistically increased compared to the control.

A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control, Canton S. ( $p \leq 0.001$ ; Control= Canton S  $n = 125$ , 91Y  $n = 50$ , lov<sup>10</sup>  $n = 100$ , lov<sup>38</sup>  $n = 50$ , lov<sup>47</sup>  $n = 100$ , lov<sup>65</sup>  $n = 50$ , lov<sup>66</sup>  $n = 50$ , lov<sup>66</sup>/CyO  $n = 50$ )

benzaldehyde was above 3, suggesting that these lines could smell and respond properly to benzaldehyde (Figure 7-7). For  $lov^{47}$ , the ORI for 1% benzaldehyde was 4.2 and the ORI for 0.1% benzaldehyde was 3.0, suggesting  $lov^{47}$  has a slight defect in its ability to smell and respond to repelling odorant (Figure 7-7). However, this difference is not statistically significant.

Finally, the ability of the various fly lines to sense and respond to pheromones was tested. The flies' ability to detect female pheromones was previously tested using feminized males as courtship objects. Those experiments are discussed in Chapter 6. It was found that the various *lov* mutants can detect female pheromones and respond to them. However, several of the lines—91Y,  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{47}$ , and  $lov^{65}$ —demonstrated abnormal behaviors during the assay and had an increase in non-directed courtship in which they displayed courtship steps but not directed at the target fly (either wild type female or feminized male). Both abnormal behaviors and the increase in non-directed courtship suggest that while the lines can detect the female pheromones and respond to them, these lines appear to have a difficulty processing and localizing the source of the pheromone. To determine if this defect is specific to female pheromones or is a more general defect to all pheromones, the smell assay was used. The odorant used is a male specific pheromone, cVA, which at low concentration is an attractant (5 to 50 ng) whereas at high concentration (500  $\mu$ g), it is a repellent (Wang and Anderson, 2010). Both males and females are attracted to cVA at the lower concentration and its purpose is to attract flies in large groups so males can more easily find suitable females to mate with. At higher concentrations, it is believed to induce aggressive behaviors, creating a

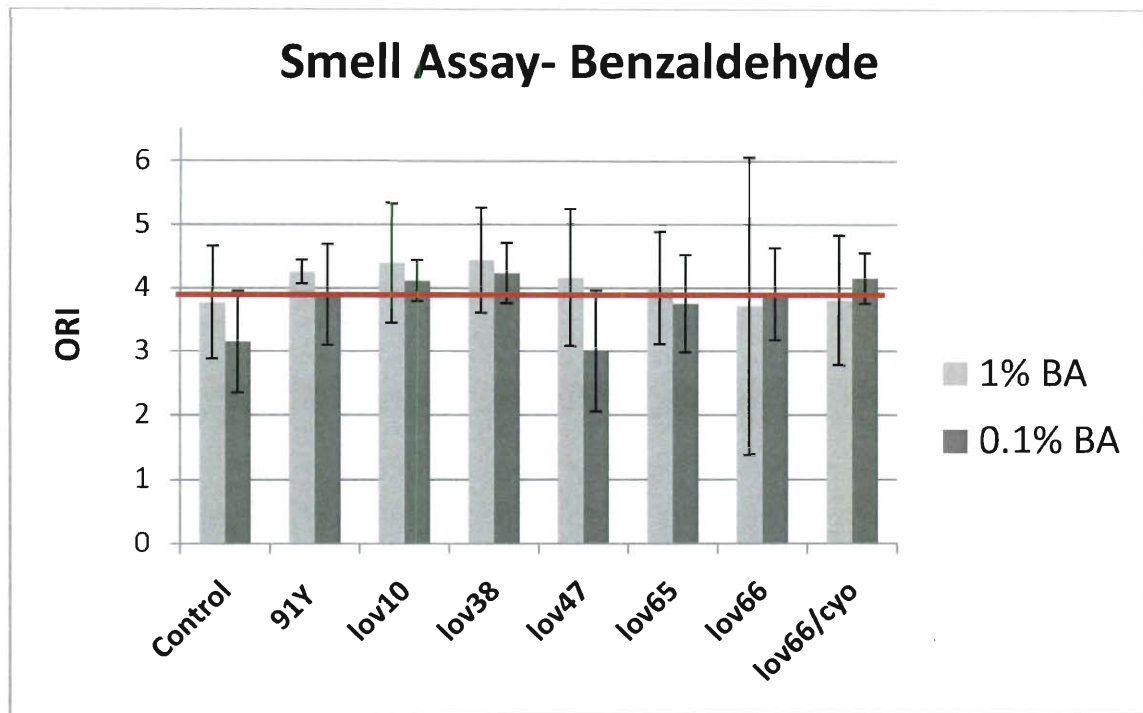


Figure 7-7: Olfactory Response Index (ORI) to 1% benzaldehyde (BA) and 0.1% benzaldehyde (repelling odorant) for the various lov mutants. An ORI above 3 (above the red line) means flies are repelled by the odorant. For all the lines except lov<sup>47</sup>, the ORI above 3 for both concentrations of benzaldehyde, suggesting that for these lines, the gross olfactory response is intact. lov<sup>47</sup> has an ORI of 3.0 when exposed to 0.1% benzaldehyde, suggesting that this line is slightly neutral to lower concentration of benzaldehyde. However, this difference is not statistically significant. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control, Ore R. (1% benzaldehyde:  $p = 0.593$ ; Control=Ore R  $n = 50$ , 91Y  $n = 50$ , lov<sup>10</sup>  $n = 50$ , lov<sup>38</sup>  $n = 50$ , lov<sup>47</sup>  $n = 50$ , lov<sup>65</sup>  $n = 50$ , lov<sup>66</sup>  $n = 50$ , lov<sup>66</sup>/CyO  $n = 50$ ; 0.1% benzaldehyde:  $p = 0.004$ ; Control=Ore R  $n = 50$ , 91Y  $n = 50$ , lov<sup>10</sup>  $n = 50$ , lov<sup>38</sup>  $n = 50$ , lov<sup>47</sup>  $n = 50$ , lov<sup>65</sup>  $n = 50$ , lov<sup>66</sup>  $n = 50$ , lov<sup>66</sup>/CyO  $n = 50$ )



negative feedback loop to maintain an optimal amount of flies for the available resources.

For this assay, solutions of 50 ng, 5 ng, and 0.5 ng cVA were used as an attractive scent. Concentrations below 0.5 ng yielded a neutral response in the control flies thus suggesting that 0.5 ng of cVA is the lowest concentration that flies can detect as attractive. For each line, at least 10 trials with five flies each were performed. If a line yielded an inconclusive result, additional trials were performed.

As can be seen from Figure 7-8, the control line, Canton S, is attracted to cVA at all three exposure levels and is most attracted to the highest concentration of cVA. Both *lov<sup>66</sup>* and *lov<sup>66</sup>/CyO* had an attractive to slightly neutral response to cVA. The ORI ranged between 2 and 2.1 for *lov<sup>66</sup>* and 1.9 to 2.1 for *lov<sup>66</sup>/CyO*, suggesting that these lines have a slight impairment in detecting and responding to cVA (Figure 7-8). *lov<sup>10</sup>* appears to be able to detect cVA at higher concentration (50 ng) but not at lower concentrations (5 ng and 0.5 ng) suggesting that this line has a reduced sensitivity to cVA and possibly to pheromones in general given that it has one of the higher levels of non-directed courtship for both wild type females and feminized males (Figure 7-8). For the remaining lines—91Y, *lov<sup>38</sup>*, *lov<sup>47</sup>*, and *lov<sup>65</sup>*—a neutral response is generated at all three amounts of cVA, suggesting that these lines are unable to detect and respond appropriately to cVA and possibly other pheromones (Figure 7-8). Combined with the increase in non-directed courtship when tested with feminized males, it appears that all of these lines have a defect in pheromone detection and response.

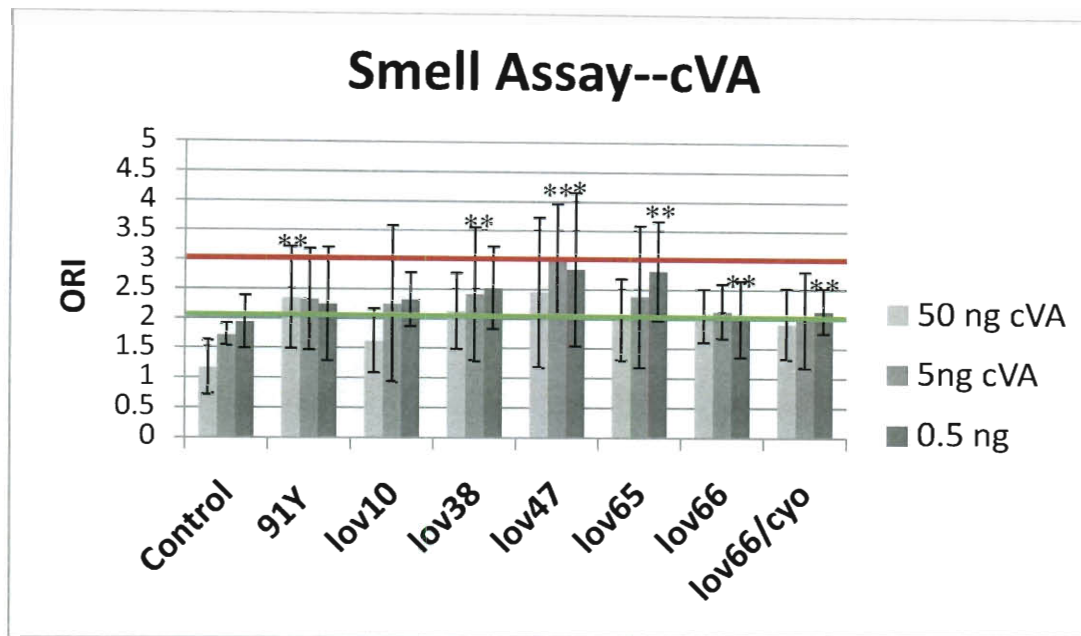


Figure 7-8: Olfactory Response Index (ORI) to 50 ng, 5ng, and 0.5 ng cVA (attractant odorant) for the various lov mutants. An ORI below 2 (below the green line) means flies are attracted to the odorant. Only the control, Canton S, is attracted to all three amounts of cVA as its ORI is less than 2 for all three levels.  $lov^{66}$  and  $lov^{66}/CyO$  have slight neutral responses to all three with ORIs ranging between 1.9 and 2.1. The remaining lines are neutral ( $2 \leq \text{ORIs} \leq 3$ ) to cVA at lower amounts of cVA and neutral ( $2 \leq \text{ORIs} \leq 3$ ) or slightly attracted ( $\text{ORIs} \leq 2$ ) at higher amounts of cVA (50 ng). A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control, Canton S. (50 ng cVA:  $p = 0.01$ ; Control=Canton S  $n = 50$ , 91Y  $n = 50$ ,  $lov^{10}$   $n = 70$ ,  $lov^{38}$   $n = 100$ ,  $lov^{47}$   $n = 50$ ,  $lov^{65}$   $n = 125$ ,  $lov^{66}$   $n = 75$ ,  $lov^{66}/CyO$   $n = 125$ ; 5 ng cVA:  $p = 0.10$ ; Control=Canton S  $n = 50$ , 91Y  $n = 50$ ,  $lov^{10}$   $n = 50$ ,  $lov^{38}$   $n = 100$ ,  $lov^{47}$   $n = 50$ ,  $lov^{65}$   $n = 50$ ,  $lov^{66}$   $n = 75$ ,  $lov^{66}/CyO$   $n = 75$ ; 0.5 ng cVA:  $p = 0.10$ ; Control=Canton S  $n = 50$ , 91Y  $n = 100$ ,  $lov^{10}$   $n = 50$ ,  $lov^{38}$   $n = 50$ ,  $lov^{47}$   $n = 50$ ,  $lov^{65}$   $n = 50$ ,  $lov^{66}$   $n = 75$ ,  $lov^{66}/CyO$   $n = 75$ )

The gross olfactory response appears to be intact for 91Y,  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{65}$ ,  $lov^{66}$ , and  $lov^{66}/CyO$  as each of these lines appear to be able to detect and respond appropriately to both water and benzaldehyde. However, each of these lines appears to have a reduction in its ability to detect and respond to cVA, suggesting a defect in their ability to detect pheromones. For  $lov^{66}$  and  $lov^{66}/CyO$ , this defect maybe limited only to detection of cVA as these flies have a similar Courtship Index to feminized males when compared to control flies and have a similar non-directed Courtship Index as controls. However, it appears that 91Y,  $lov^{10}$ ,  $lov^{38}$ , and  $lov^{65}$  all have defects in detecting and responding to pheromones in general. Each of these lines had a decreased Courtship Index when exposed to both wild type females as compared to the control, an increase in non-directed courtship as compared to the control for both females and feminized males, and a neutral response to cVA, especially at higher amounts of cVA. This suggest for 91Y,  $lov^{10}$ ,  $lov^{38}$ , and  $lov^{65}$ , the observed defects in courtship may be due in part to a defect in their ability to detect and respond to pheromones.  $lov^{47}$  appears to have a general defect in olfactory response. It is slightly repelled by water, slightly neutral to lower concentrations of benzaldehyde, neutral to all three amounts of cVA, and has the highest level of non-directed courtship among the various *lov* mutants. This impaired olfactory response may be responsible for its courtship defect. Given that 91Y is the base chromosome for all the mutants, it is not unexpected that most of the mutants have a similar olfactory defects as 91Y.

Is the non-directed courtship behavior observed in some of the *lov* mutants due to a defect in vision?

As mentioned above, several of the *lov* mutant lines, 91Y,  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{47}$ , and

lov<sup>65</sup>, demonstrate a non-directed courtship behavior in which they displayed various courtship steps, primarily wing extension, but not directed at the target fly (either wild type female or feminized male). A modified smell assay and courtship experiments using feminized males as courtship targets determined that these lines have defects in detecting and responding to pheromones properly. However, this does not rule out the possibility these mutant have defects in vision or in processing visual information and that this is what is responsible for the non-directed courtship behavior. There is no known expression of lov in the eye, therefore if there is a defect in vision; it is most likely in the processing of visual information rather than in the receiving of visual information.

As can be seen from Figure 7-10, for all the lines tested at close to 70% of the flies chose the tube exposed to light. This suggests that gross vision and response to visual input is normal in all of the lov mutant lines. Therefore, it is unlikely that defects in vision and visual processing are responsible for the observed courtship defects.

Do the various lov mutants have defects in motility that affect their ability to court properly?

When the various mutants were tested in the courtship assay, it was observed that at least two of the lines, lov<sup>38</sup> and lov<sup>47</sup>, were sluggish in the courtship assay. In particular, lov<sup>47</sup> males appeared to only make attempts to court females when they are in close proximity. lov<sup>38</sup> males appear to be fairly active when in a vial by themselves or when initially placed in the courtship chamber before the female is introduced. However, once the female is in the courtship chamber, the activity level of the male

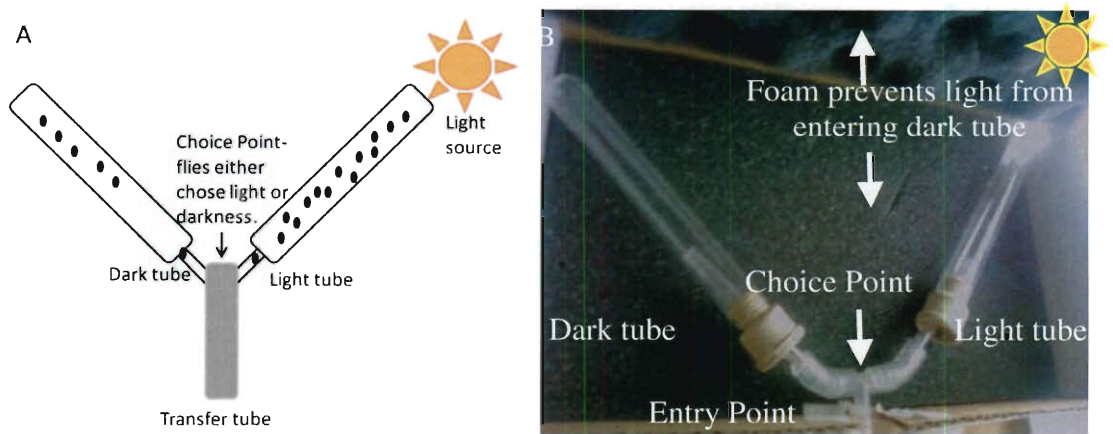


Figure 7-9: Vision Assay set-up. The flies were placed in a t-test apparatus in which one tube is exposed to a light source and one tube remains in the dark. The flies were then allowed one minute to choose which tube to enter. A. Schematic drawing of the t-test apparatus. B. Photograph of actual t-test apparatus used in the vision assay.

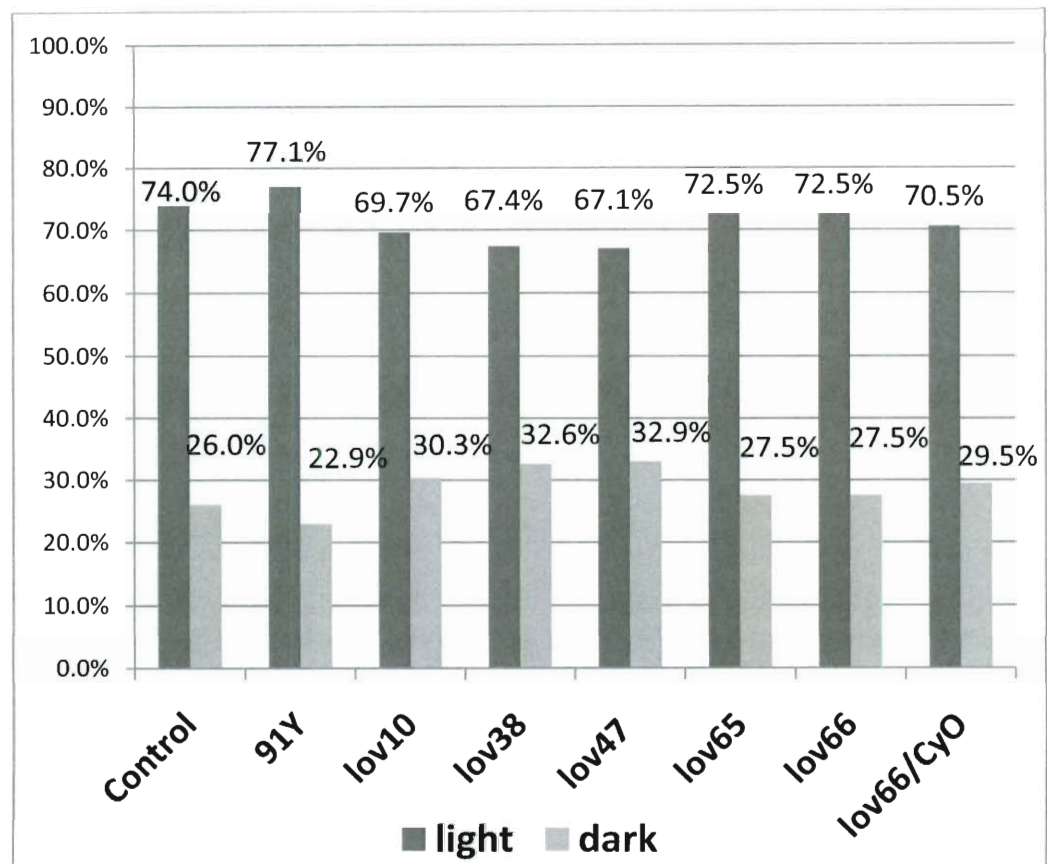


Figure 7-10: Vision Test for various lov mutants. If gross vision and response to visual input is normal, the majority of flies for any given line will chose the tube that is exposed to light. For all of the lov mutant lines, the majority of the flies chose the light tube, suggesting that their vision and response to visual input is normal. A chi-squared test was performed for each line versus the control to determine if there was a statisicaly significant difference for any of the lines. (Control= Canton S n=100, 91Y n=170, lov<sup>10</sup> n=145, lov<sup>38</sup> n=132, lov<sup>47</sup> n=82, lov<sup>65</sup> n=138, lov<sup>66</sup> n=138, lov<sup>66</sup>/CyO n=146;  $\chi^2_{CS, 91Y}$  =0.827;  $\chi^2_{CS, lov10}$  = 1.42;  $\chi^2_{CS, lov38}$  =2.97;  $\chi^2_{CS, lov47}$  =2.04;  $\chi^2_{CS, lov65}$  =.169;  $\chi^2_{CS, lov66}$  =.169;  $\chi^2_{CS, lov66/CyO}$  =.904)

decreased. Given the low CI of these two mutants, it is possible that they may have a motility defect that has not been previously observed. It is also possible that  $lov^{38}$  males change their activity level in response to the presence of the females.

To determine whether or not any of the  $lov$  mutants have a defect in motility and what effect the presence of other flies has on the activity level of the mutant flies, three activity assays were performed. In the first version of the assay, seven day old individual males are placed in small circular chambers that have a line dividing them in half. Each male is observed for ten minutes and the number of time the males cross the line is recorded (Figure 7-11a). Both the fly's age and the observation period matched the courtship assay conditions, allowing for an easier comparison between the behaviors in the two assays. The second assay involves placing a seven day old  $lov$  mutant male and four day old wild type male in the same chamber and again observing the mutant male's activity level for 10 minutes (Figure 7-11b). This assay will allow us to determine if the activity level of a particular mutant decreases in the presence of another fly. Finally, the third assay will pair a seven day old  $lov$  mutant with a four day old wild type female, and the activity level of the male is recorded (Figure 7-11c). This assay will allow us to determine if the presence of the female decreases the activity level of the mutant males.

As can be seen in Figure 7-12, the activity level of males when placed by themselves in the courtship chamber varied among the lines. The control line, Ore R and 91Y had the lowest activity levels. Yet these two lines have among the highest Courtship Index. On the other hand,  $lov^{38}$ , which has the lowest Courtship Index, has the highest activity level. It would appear then that the activity level of an individual fly by itself has no correlation to its ability to court. Therefore, it is unlikely that a motility defect is

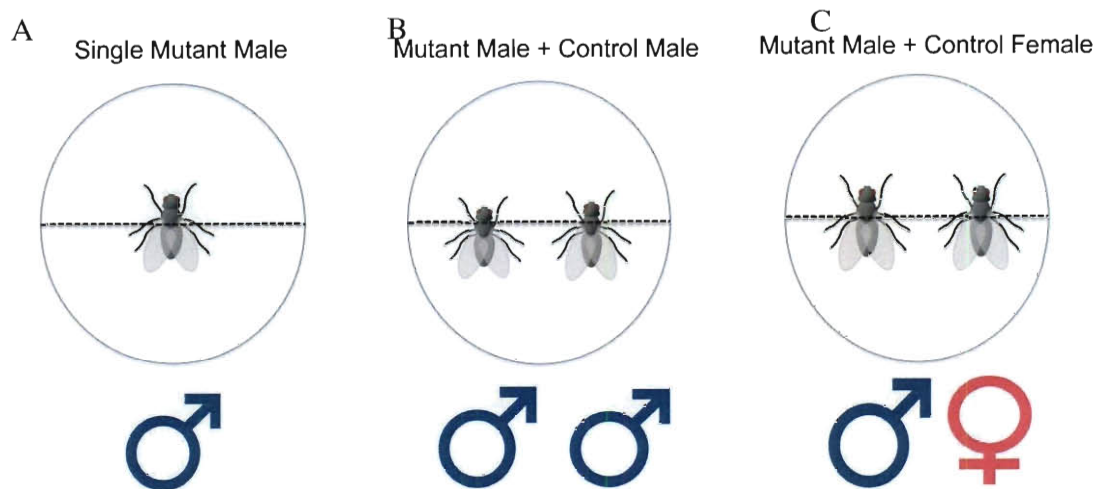


Figure 7-11: The three activity assays. A. In the first assay, a single male is placed in the courtship chamber by himself and the number of times he crosses the center line is record. B. In the second assay, the mutant male is place in the courtship chamber with a control male, *Sco/Cyo*, to determine how the fly's behavior is altered in the presence of another fly. C. In the third assay, a male is place in a courtship chamber with a control female to determine how the fly's behavior is altered in the presence of a female fly and if the response is different compared to another male fly in the chamber.



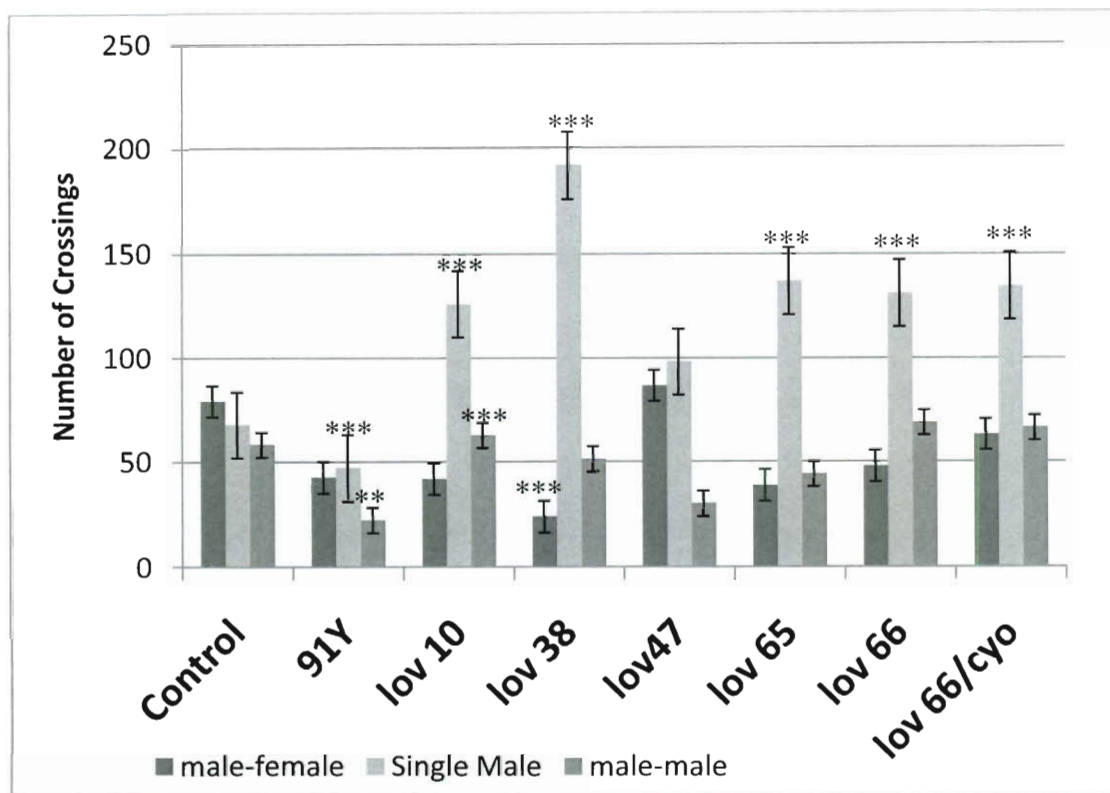


Figure 7-12: Activity levels for the various lov mutants under three different conditions. In the single fly assay, the control line, Ore R and 91Y had the lowest activity levels.  $lov^{38}$ , which has the lowest Courtship Index, has the highest activity level. In the male-male assay, the activity level for all the lines dropped with the greatest decrease in activity levels observed in  $lov^{47}$  and 91Y. In the male-female assay, activity levels of  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{65}$ ,  $lov^{66}$ , and  $lov^{66}/CyO$  decreased significantly compared to the single fly condition and to a lesser extent to the male-male condition. For Ore R,  $lov^{47}$  and 91Y, the activity level was greater in the presence of the female as compared to another male and was similar or greater than the activity level of male by itself. A one-way ANOVA was performed to determine if there were differences among the lines. A Dunnett's Test was used to compare the results of the mutant lines to the control, Canton S. A one-way ANOVA was performed to determine if there were differences among the lines.

(Figure 7-12 cont.) A Dunnett's Test was used to compare the results of the mutant lines to the control, Canton S. (Male alone:  $p \leq 0.001$ ; Control=Ore R  $n=26$ , 91Y  $n=26$ ,  $lov^{10}n=50$ ,  $lov^{38}n=50$ ,  $lov^{47}n=28$ ,  $lov^{65}n=50$ ,  $lov^{66}n=50$ ,  $lov^{66}/CyO$   $n=50$ ; Male-male:  $p=0.002$ ; Control=Ore R  $n=25$ , 91Y  $n=25$ ,  $lov^{10}n=25$ ,  $lov^{38}n=25$ ,  $lov^{47}n=25$ ,  $lov^{65}n=25$ ,  $lov^{66}n=25$ ,  $lov^{66}/CyO$   $n=25$ ; Male-female:  $p \leq 0.001$  Control=Ore R  $n=20$ , 91Y  $n=10$ ,  $lov^{10}n=19$ ,  $lov^{38}n=10$ ,  $lov^{47}n=10$ ,  $lov^{65}n=19$ ,  $lov^{66}n=10$ ,  $lov^{66}/CyO$   $n=10$ )

responsible for the observed courtship defects.

When males from the various lines were placed in the courtship chamber with another male, the activity level for all the lines dropped (Figure 7-12). The activity levels of  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{65}$ ,  $lov^{66}$ , and  $lov^{66}/CyO$  were comparable to the control, Ore R. There was a greater decrease in activity levels for  $lov^{47}$  and 91Y. The results suggest that all the lines modify their behavior in the presence other flies and that  $lov^{47}$  and 91Y have the greatest level of modification.

Finally, when the various mutant males were placed in a courtship chamber with control female fly, activity levels of  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{65}$ ,  $lov^{66}$ , and  $lov^{66}/CyO$  decreased significantly compared to the single fly condition and to a lesser extent to the male-male condition (Figure 7-12). The greatest level of decrease was observed in  $lov^{38}$ . For Ore R,  $lov^{47}$  and 91Y, the activity level was greater in the presence of the female as compared to another male and was similar or greater than the activity level of male by itself (Figure 7-12). These results suggest that the presence of another fly affects the activity level of the fly. For  $lov^{10}$ ,  $lov^{38}$ ,  $lov^{65}$ ,  $lov^{66}$ , and  $lov^{66}/CyO$ , the presence of a female fly decreases the activity level and may be partially responsible for the observed courtship defects. For 91Y, the activity level for all three conditions is lower than wild type, yet it has one of the highest Courtship Index of the various *lov* mutants. Therefore, it is unclear if this decreased level of activity is responsible for the observed courtship defect.  $lov^{47}$  behavior in the three activity assays is similar to that of Ore R. Therefore, it is unlikely that the observed courtship defect is due to a decrease in activity.

Are other behaviors which are not related to courtship affected by the various *lov* mutations?

Unlike  $lov^{66}$ , the other four mutants initially demonstrated no obvious phenotype.

However, because these lines were generated from 91Y, which is known to have a defect in gravitaxis, additional behavioral experiments were performed to see if any of the mutants have defects in gravitaxis.

*lov*<sup>47</sup> exhibits altered climbing behavior

The Climb Test was used to determine if any of the *lov* mutants have a locomotor defect. This examines the locomotor ability of a fly after a stressor is applied. In this test, a fly is gently tapped to the bottom of an empty vial, and the time for the fly to climb to 5 cm is recorded (Figure 7-13). Wild type flies will climb straight up the side of the vial and reach the 5 cm mark in about 3 seconds.

*lov*<sup>10</sup>, *lov*<sup>38</sup>, *lov*<sup>65</sup>, and *lov*<sup>66</sup> have climb times close to the control, 91Y, suggesting that these mutants have a normal locomotor ability (Figure 7-14). For all of these lines, the average climb time is about 3.5 seconds. On the other hand, *lov*<sup>47</sup> has a much slower time of 5.6 seconds, taking almost twice as long. This suggests that *lov*<sup>47</sup> has a locomotor defect under stressful situations.

*lov*<sup>38</sup> and *lov*<sup>47</sup> demonstrate altered behavior in the Gravitaxis Maze Assay

The original *lov*<sup>38</sup> and *lov*<sup>47</sup> excision mutants had no obvious phenotypes, but because they were generated from 91Y, both lines were tested in the maze as part of their characterization. The maze assay was previously described in the introduction and is used to identify flies which have altered gravitactic responses (Figures 1-1 & 1-3). These assays were performed using the original excision mutant lines, not in the isogenic background, and thus the flies tested had *w*<sup>-</sup> eyes, which may have altered their behavioral response in the maze. However, their behavior in the mazes is very different to the *w*<sup>-</sup> flies (Beckingham, personal communication). Repeating these experiments using the isogenic

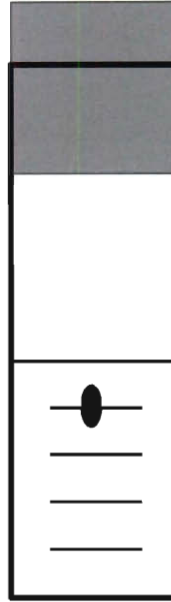


Figure 7-13: Set up for the climb test. Flies are placed into individual empty food vials which had marks every 1 cm up to 5 cm. The flies were then gently tapped and observed for 60 seconds to determine how long each fly takes to climb to the 5 cm mark.

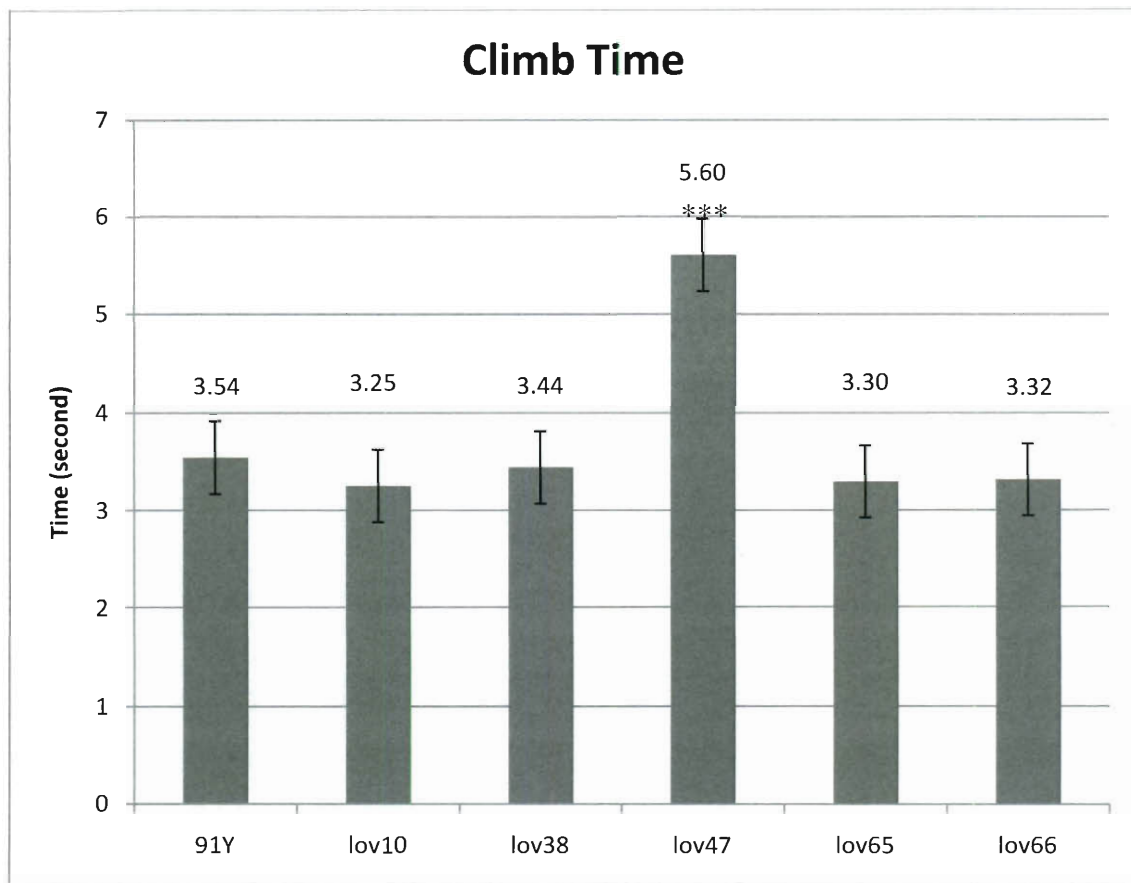


Figure 7-14: Climb Test results for lov mutants. For most of the lov mutants, the time it takes to climb to 5 cm is about 3.5 seconds, similar to the control, 91Y. In contrast, lov<sup>47</sup> has a climb time of 5.6 seconds, suggesting that lov<sup>47</sup> locomotor defect after a stressor is applied. A one-way ANOVA and a Dunnett's Test were performed to determine statistical significance. ( $p \leq 0.01$ ; 91Y n=54; lov<sup>10</sup> n= 70; lov<sup>38</sup> n= 61; lov<sup>47</sup> n=50; lov<sup>65</sup> n=55; lov<sup>66</sup> n=50)

flies may be performed at a later date, along with the remaining *lov* mutants.

For both lines, when the flies were allowed to run the maze for three hours (the standard run time), none of the flies exited the maze (Figure 7-15a and b). In addition, the flies that had made it into the maze remained near the entrance and were on the lower half of the maze. When the flies were allowed to run the maze for 24 hours, most of the flies completed the maze. Those flies that exited from the maze, did so at the bottom of the maze (Figure 7-15c and d). The results suggest that both lines have a gravitaxic defect under normal conditions. Additionally, other behaviors appear to be affected in these lines as it takes both lines much longer time to complete the maze compared to wild type. Wild type flies will complete the maze in 3 hours.

The altered behavior in both the Gravitaxis Maze Assay and the Climb test suggests that *lov* function in behaviors outside of courtship. Therefore *lov* functions to specify many behaviors in *Drosophila* and may function to set up the different neural circuits required for different behaviors. Given that there are multiple predicted transcripts of *lov* and EYFP expression has shown differential expression in males and female, it is possible that the different transcripts of *lov* help to specify different behaviors in *Drosophila*. Additional behaviors should be examined in the various *lov* mutants to determine what other behaviors *lov* is involved in.

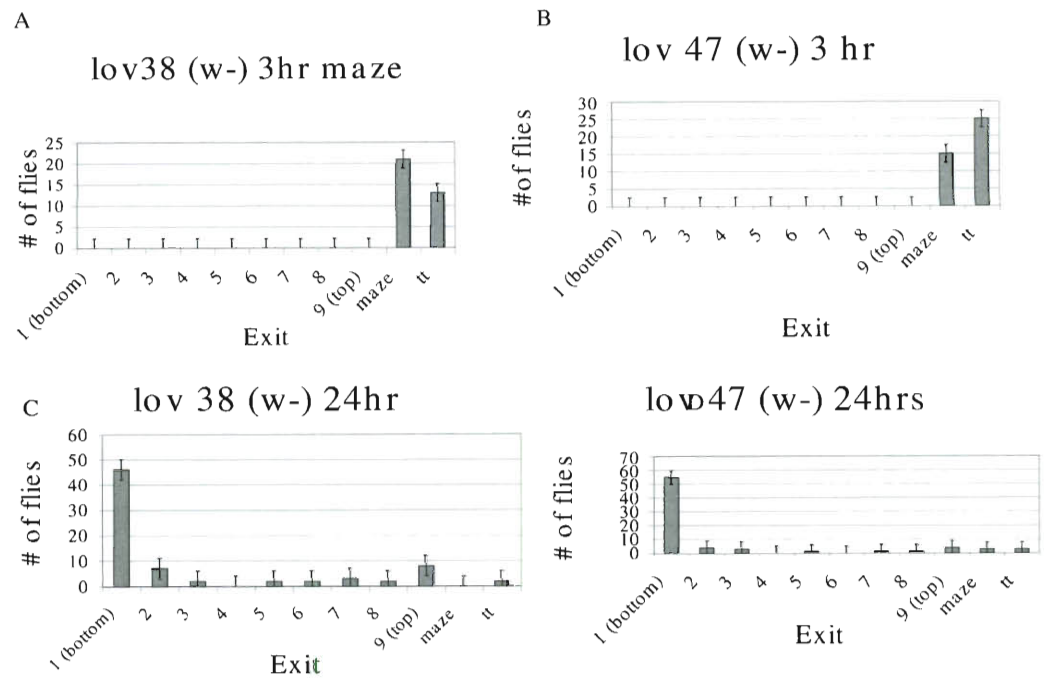


Figure 7-16: Maze Data for  $lov^{38}$  and  $lov^{47}$ : A. 3 hr Maze data for  $lov^{38}$  shows that none of the flies exit the maze after 3 hrs. The flies were either had entered the maze or remained in the transfer tube (labeled as tt on the charts) B. 3 hr Maze data for  $lov^{47}$  shows that none of the flies exit the maze after 3 hrs, similar to  $lov^{38}$ . Again, the flies were either had entered the maze or remained in the transfer tube (labeled as tt on the charts) C. 24 hr Maze data for  $lov^{38}$  shows that the flies exit the maze after 24 hrs and that  $lov^{38}$  is a low line. D. 24 hr Maze data for  $lov^{47}$  shows that the flies exit the maze after 24 hrs and that  $lov^{47}$  is again a low line.



## Material and Method

### Proboscis Extension Response (PER) assay

To perform this assay, newly eclosed male flies were collected and placed in a food vial containing only a moistened kimwipe and starved overnight. The following morning, the flies were mounted onto toothpicks by tacking down their wings with Tissue Tack. The flies were then allowed to recover for three hours. Prior to the introduction of the tastants, the flies were offered water to satiation to ensure the response in the assay is to the tastant introduced and not to a behavioral response to water alone. Next the various tastants, 4% sucrose, 1% sucrose (both attractants), or 4% caffeine + 4% sucrose (repellent) were touched to the front leg. Each fly was tested three times with water offered between testing. A PER score of one was given if a fly extended all three times. A PER score of zero was given if a fly did not extend all three times. If the fly gave a mixed response in the first three trials, two additional trials were performed. After five trials, if a fly extended three or more times, a PER score of one was given. Otherwise, a PER score of zero was given. Approximately 50 flies from each line were tested.

### Larval Test Assay

To perform this assay, embryos from the various lov lines were collected on yeasted grape plates and allowed to age for two days. On the evening of the second day, the larvae were then transferred to a plain agarose plate and starved until morning. The following morning, approximately fifty, three-day-old larvae were transferred to experimental plates and allowed to wander on the plates for 15 minutes in the dark. Two types of experimental plates were used. The first set of plates tested the larval response to the attractant. A large petri dish was divided in half with one half containing only agarose and the other half containing agarose and 4% sucrose. If the larvae on this plate can taste

and respond properly, the majority of the larvae would be on the side containing the sucrose. The second set of plates was likewise divided in half. This time, one half of the plate contained agarose and the other half contained agarose, 4% sucrose, and 4% caffeine. This set of plates tested the larval response to the repellent. If the larvae on this plate can taste and respond appropriately, the majority of larvae should be on the side that contains only agarose. To score this assay, the number of larvae that were more than 0.5 cm from the junction of control (plain) agarose and experimental (sucrose or sucrose plus caffeine) agarose were counted. The number on the control side (agarose) was subtracted from the number on the experimental side (sucrose or sucrose plus caffeine) and the resulting number was divided by the total number of larvae to provide the normalized score, which is the larval response index (LRI). If on the sucrose plates, the LRI was positive, it means that the larvae can taste and respond appropriately to sucrose. If the LRI is negative on the sucrose plate, it means that the larvae cannot taste and/or respond appropriately to sucrose. If the LRI is negative on the sucrose plus caffeine plate, it either means that the larvae cannot taste and/or respond appropriately to caffeine or are attracted to caffeine when they should be repelled by it.

#### Smell Assay

In this assay, males were collected and allowed to age four to six days. They were then placed in empty food vials in groups of five. A Q-tip pre-soaked with 100  $\mu$ l of neutral, repellent, or attractive odorant was then placed in the vial so that the tip of it was in the middle of the vial. For one minute, the number of flies at the far end of the vial (opposite the plug) was counted every five seconds. The number of flies at the far end was averaged to give an Olfactory Response Index (ORI). The assay was repeated at least

ten times so a minimum of 50 flies were tested. If the flies are evenly distributed throughout the vial, the line is neutral to the odorant and has an ORI between two and three. If the majority of the flies ( $>3$ ) were at the far end of the vial, it means that line is repelled by the odorant and has an ORI above three. If the majority of the flies were in the half of the vial containing the odorant (near the plug) ( $>3$ ) it means that line is attracted to the odorant and the line has an ORI less than two.

### Vision Assay

To test for gross defect in vision, the various lines were tested for their ability to detect and respond to light (Figure 7-9). The flies were placed in a t-test apparatus in which one tube is exposed to a light source and one tube remains in the dark. The flies were then allowed one minute to choose which tube to enter. Wild type flies are attracted by light and the majority of the flies will go into the tube that is exposed to light. If a mutant line has defects in vision or visual processing, the flies in those lines would be neutral to light and the flies would be evenly distributed between the light and dark tubes or attracted to dark and the majority of the flies would be in the dark tube. For this assay, vials of 25 newly eclosed males were collected and allowed to age for 3 to 5 days. The flies were then transferred to a dark transfer tube and the transfer tube was attached to the t-test apparatus. A light source was then turned on to expose one of the tubes to the light. The flies were allowed one minute to make the choice of either the light tube or dark tube. After the minutes was up the number of flies in the dark tube and the light tube were counted and recorded. The assay was repeated six times so approximately 150 males were tested. The lines tested with this assay were Canton S (wild type control), 91Y (original *lov* mutant), *lov*<sup>10</sup>, *lov*<sup>65</sup>, *lov*<sup>38</sup>, *lov*<sup>47</sup>, *lov*<sup>66</sup>, and *lov*<sup>66</sup>/CyO.

### Climb Test

In this assay, newly eclosed males were collected, placed into individual vials and allowed to age two days. They were then placed into individual empty food vials which had marks every 1 cm up to 5 cm. The flies were then gently tapped and observed for 60 seconds to determine how long each fly takes to climb to the 5 cm mark. The flies were each tested 10 times with a 60 second recovery period between trails. Approximately 50 flies from each line were tested.

### Gravitaxis Maze Assay

For this assays, vials of 25 newly eclosed males were collected and allowed to age for 2 days. The flies were then transfer to a dark transfer tube and the transfer tube was attached to the maze apparatus. The maze was then placed in a box so that the entire maze was dark with the exception of the collection tubes at the far end of the maze. A light source was then turned on to expose collection tubes to the light. The collection tubes contain a small amount of yeast paste to encourage the flies to move through the maze. The flies were allowed three hours to navigate the maze. After the three hours were up, the number of flies in the exit tubes were counted and recorded. The assay was repeated four times so approximately 100 males were tested. A second set of maze assays were repeated with the amount of time the flies were allowed to navigate the maze increased to 24 hours to allow the maximum number of flies to exit the maze.

### Statistical Analysis

For the adult taste assays and the vision assay, a chi-squared test was used to determine if there were differences among the lines. For the remaining assays, a one-way ANOVA was performed to determine if there were differences among the lines. A

Dunnett's Test was used to compare the results of the mutant lines to the control line. An alpha of 0.05 was used for all statistics. The number of males (n) tested and the p-value is listed in each figure legend. Statistical significant is indicated in each figure in the following way: \* =  $p \leq 0.05$ ; \*\* =  $p \leq 0.01$ ; \*\*\* =  $p \leq 0.001$ .

## CHAPTER 8: ALTERED LOV EXPRESSION EXPERIMENTS TO EXAMINE lov FUNCTION

As discussed in Chapters 6 and 7, the lov mutants have behavioral defects in courtship, olfaction, and gustation, suggesting that lov has a function in these behaviors. To further examine the function of lov, two sets of mis-expression experiments were performed using the GAL4/UAS system (Figure 1-2). These experiments enabled us to investigate whether the mis-expression of lov could produce similar phenotypes to those observed in the lov mutants, thus helping to confirm lov's function in those behaviors as well as to identify new potential functions of lov.

For the first set of experiments, two constructs were prepared in which the full length lov protein coding region was inserted into the Drosophila vector pUAST. One of these constructs fused Lov with a fluorescent protein to allow for easier visualization of the lov expression pattern. Various neuronal GAL4 drivers were used to drive expression of lov in the resulting UAS-lov lines. This resulted in both over-expression of lov in some neurons and ectopic expression of lov in others. Throughout the rest of the chapter, the term over-expression was used to discuss both the over-expression and ectopic expression of lov.

In the second set of experiments, lov expression was reduced through the use of a lov RNAi transgene. A series of neuronal GAL4 drivers were used to selectively reduce lov levels in different neurons. Throughout the rest of the chapter, the term under-expression was used to discuss the reduced expression of lov in the different neurons produced with this transgene.

### Generation of UAS-lov and UAS-lov-mCherry transgenic lines

To generate a pUAST-lov construct, the coding region of lov from lov cDNA

G08221, which contains the entire protein coding region, was amplified using PCR. The resulting PCR product was inserted into the pUAST vector using standard cloning techniques (see Materials and Methods). Transgenic larvae were prepared as described in Material and Methods and lines carrying single insertions on a particular chromosome were prepared as shown in Figures 8-1 and 8-2.

A second lov transgenic construct was generated in which lov is tagged at its C terminus with the red fluorescent protein, mCherry (Campbell et al., 2002). mCherry was chosen because of its strong signal and its resistance to photobleaching. This construct would allow for visualization of lov in over-expression experiments.

To generate this construct, the coding region of lov was amplified with PCR using the lov cDNA G08221. Likewise, the coding region of mCherry was amplified with PCR using the mCherry vector (Clontech). The lov PCR reaction introduced a linker that would lie between lov and mCherry so that both proteins could fold properly without interference from one another. The resulting PCR products were inserted into the pUAST vector using standard cloning techniques (see Materials and Methods). Transgenic larvae were prepared as described in Material and Methods and lines carrying single insertions on a particular chromosome were prepared as shown in Figures 8-1 and 8-2.

The end result for UAS-lov was one insertion line on the first chromosome, five insertion lines on the second chromosome (two lethal lines), and three insertion lines on the third chromosome (one lethal line). The end result for UAS-lov-mCherry was one insertion line on the first chromosome, seven insertion lines on the second chromosome (two lethal lines), and six insertion lines on the third chromosome (one lethal line).

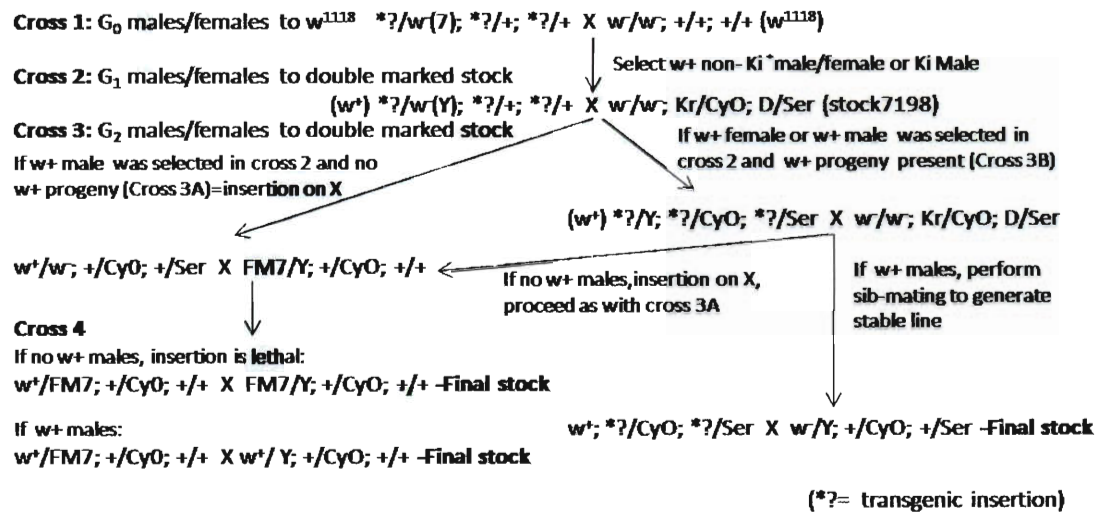


Figure 8-1: Crossing scheme to generate lov transgenic lines. The logic used to determine if insertions of the UAS-lov and UAS-lov-mCherry transgenes are on the X chromosome is indicated.



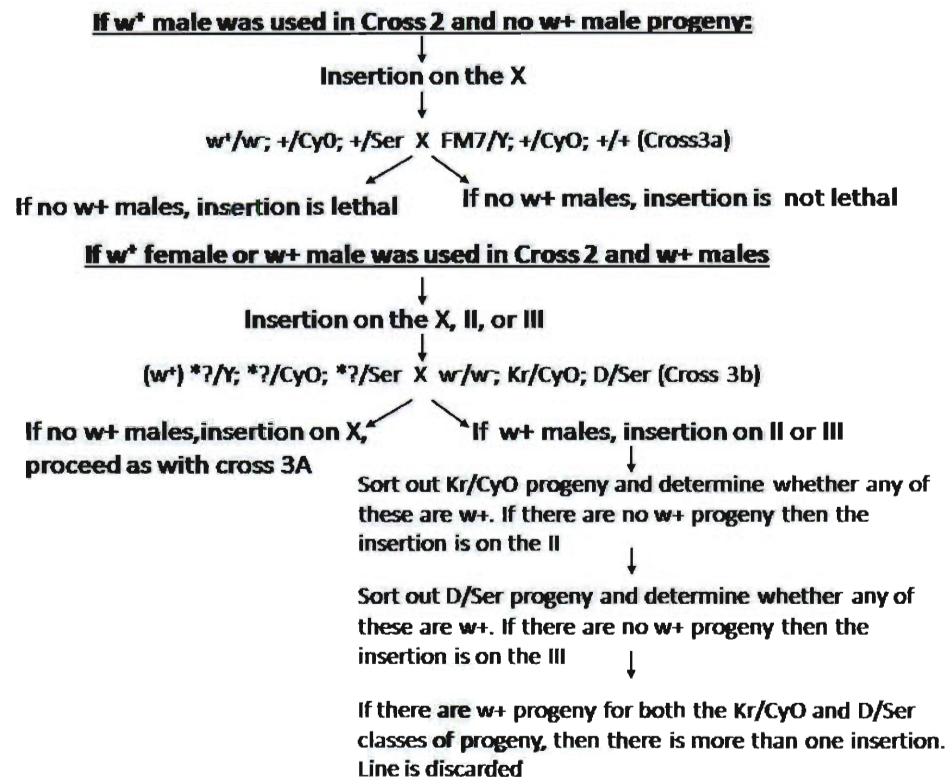


Figure 8-2: Mapping scheme to determine location of *lov* transgenes. The logic used to determine the location of a UAS-*lov* or UAS-*lov*-mCherry transgene insertion is indicated

## Over-expression experiments using UAS-lov and UAS-lov-mcherry

Once stable stocks were established and the locations of the insertions for both constructs were identified, the lines were crossed to various GAL4 lines to determine the effects of lov over-expression throughout development.

Two UAS-lov-mCherry lines and three UAS-lov lines were chosen for the over-expression experiments. All five lines have the transgene inserted on the second chromosome. The lines vary in their strength of expression (Table 8-1). An unexpected consequence of the tagged-lov lines is that the mCherry tag appears to moderate the effect of lov mis-expression.

Six GAL4 lines were used in the over-expression experiments. The expression pattern ranged from expression throughout the entire nervous system to a small subset of neurons from the known lov expression pattern (Table 8-2). The range of expression patterns allowed for the examination of the effect of lov over-expression in different neurons types and assessment of how lov might alter behavior when expressed in neurons know to be important for particular behaviors.

The effects of over-expression using the GAL4 drivers were assessed in progeny from a cross of a line carrying the GAL4 driver with a line homozygous for a UAS-lov construct. Progeny were assessed for: i.) normal embryonic development; ii.) normal larval development; iii.) normal adult morphology and fertility; and iv.) where possible, specific behavioral responses in adults. Details of analysis for the various stages can be found in Material and Methods.

## lov over-expression with elav-GAL4

To examine the effects of lov over-expression in the entire nervous system, the

UAS-lov line	Chromosome	Strength of Expression
UAS-lov4-mCherry	Second	Weak*
UAS-lov20-mCherry	Second	Moderate*
UAS -lov22	Second	Strong
UAS -lov37	Second	Strong
UAS -lov14	Second	Strong

**Table 8-1: UAS-lov lines used for lov over-expression experiments**

\*It is believed that the mCherry protein vitiates to some extent the effect of the over-expression of lov.

GAL4 Driver	Chromosome	Expression Pattern
elav-GAL4 (X)	First	Every neuron*
elav-GAL4 (II)	Second	Every neuron**
elav-GAL4 (III)	Third	Every neuron**
P0163	Third	Every neuron in PNS
atonal-GAL4	Third	Chordotonal organs
fruM-GAL4	Third	In neurons expressing male- specific form of fru

Table 8-2: GAL4 lines used in over-expression experiments

\*dosage compensation on X chromosome results in twice as much expression of GAL4 in males than females; therefore any effect due to lov over-expression would be greater in males.

\*\*elav-GAL4 expression in this line is believed not to capture the entire elav expression pattern and therefore effects of lov over-expression may be less when this line is used as compared to the elav-GAL4 (X) line.

various UAS-lov lines were crossed with three elav-GAL4 lines. The first line tested was elav-GAL4 (X). Because males only have one X chromosome whereas females have two, dosage compensation occurs in which genes on the X chromosome express twice as strongly in males. Therefore, males will have twice the level of GAL4 found in females, leading to a higher expression level of UAS-lov. Thus, it would be expected that any effect of lov over-expression would be greater in males than in females.

When the embryos from these crosses were examined, it was found that the elav-GAL4(X)/UAS-lov4-mCherry and the elav-GAL4(X)/UAS-lov20-mCherry embryos developed normally and were able to hatch into larvae (Table 8-3). In contrast, none of the elav-GAL4(X)/UAS-lov37, elav-GAL4(X)/UAS-lov22, or elav-GAL4(X)/UAS-lov14 embryos hatched. When the embryos were examined, it was found that 75% to 92% of the embryos were in advanced stages of development (Table 8-3). For many of the unhatched eggs after the chorion layer was chemically removed, a fully formed live larva was found inside. However, the movements of the larvae were uncoordinated and the larvae died shortly after the removal of the chorion. This difference between over-expression of Lov protein alone or Lov tagged with mCherry suggest that mCherry vitiates, to some extent, the effect of the over-expression of lov. This possibility is supported by the finding that when elav-GAL4(X)/UAS-lov4-mCherry and the elav-GAL4(X)/UAS-lov20-mCherry embryos are immunostained with the Lov antibody, the Lov-mCherry ectopically expressing neurons in additional neurons is not detected whereas when elav-GAL4(X)/UAS-lov22 embryos are immunostained with the Lov antibody, the ectopic Lov protein is detected.

UAS- lov Line	Embryo phenotype	Adult phenotype	Courtship
UAS-lov4-mCherry	None	Males can't eclose, 20% of females eclose and 100% of eclosed females fail to expand wings	N/A
UAS-lov20-mCherry	None	Males can't eclose, 6% of females eclose and 100% of eclosed females fail to expand wings	N/A
UAS -lov37	0% hatch rate with 92% advanced stage of development	N/A	N/A
UAS -lov22	0% hatch rate with 75% advanced stage of development	N/A	N/A
UAS -lov14	0% hatch rate with 75% advanced stage of development	N/A	N/A

Table 8-3: Results of Lov over-expression experiments with elav-GAL4 (X)

The resulting larvae of the *elav-GAL4(X)/UAS-lov4-mCherry* and the *elav-GAL4(X)/UAS-lov20-mCherry* genotypes were followed throughout their development. For both lines, the larvae developed to pupation normally. However, males were unable to eclose from their pupal cases. Females were able to eclose but were unable to expand their wings and were uncoordinated in their movement (Figure 8-3).

The above results suggest that when *lov* is expressed in all neurons, the over-expression affects a sets of neurons important for the both movements required for hatching from the egg and eclosion from the pupal case. It is possible that the neurons affected by the *lov* over-expression coordinate the required movements for hatching and eclosion. Additionally, the over-expression of *lov* appears to affect a fly's ability to expand its wings, suggesting that *lov* may play a role in that behavior.

Next, *lov* over-expression was driven using *elav-GAL4(II)* on the second chromosome. The insertion for this line is lethal and is kept over a *CyO* balancer. Since all of the *UAS-lov* lines used for this experiment are on the second chromosome, the resulting offspring from the crosses will be a mixture of *elav-GAL4(II)/UAS-lov* and *UAS-lov/CyO*, but it is not possible to determine if the embryos have both *elav-GAL4(II)* and *UAS-lov* or just *UAS-lov* alone. Therefore, to examine any potential lethality of embryos in this cross, the *CyO* balancer was exchange for a *CyO-GFP* balancer. Thus, the embryos that are *elav-GAL4(II)/CyO-GFP* embryo fluoresce green whereas the *elav-GAL4(II)/UAS-lov* embryos would do not. Only the non-fluorescent embryos were examined for development.

As can be seen from Table 8-4, for *elav-GAL4(II)/UAS-lov4-mCherry* and *elav-GAL4(II)/UAS-lov20-mCherry*, there is reduced fertility. When sib-mated, females from



Figure 8-3: Examples of *elav-GAL4(X)/UAS-lov20-mCherry* pupae failing to eclose. Most of the males from *elav-GAL4(X)/UAS-lov20-mCherry* fail to eclose. Some will make it halfway out. Females manage to eclose (center fly) but fail to expand their wings properly.



these lines can lay fertilized eggs; however most of the embryos die before hatching. For elav-GAL4(II)/UAS-lov20-mCherry, those embryos that survive to adulthood are unable to expand their wings. Sib-mated flies from elav-GAL4(II)/UAS-lov22 are unable to produce viable offspring. These results suggest that when lov is overexpressed in adults, it affects their fertility. The over-expression of lov can be lethal in embryogenesis suggesting that lov has an important role in development.

Finally, over-expression of lov was driven by elav-GAL4(III) on the third chromosome. This GAL4 line is believed not to capture the entire elav expression pattern; therefore, the effects of lov over-expression may be less when this line is used as compared to the other elav-GAL4 drivers. As can be seen from Table 8-4, over-expression of lov driven by elav-GAL4(III) has less of an affect than the elav-GAL4 line on the other chromosomes. There is no obvious embryonic phenotype. The flies survived to adulthood and most of the flies appeared physically normal. Two of the elav-GAL4(III)/UAS-lov20-mCherry flies had wings that failed to expand and one fly failed to fully eclose.

However, when the elav-GAL4(III)/UAS-lov males were tested in courtship assay as described in Chapter 6, lov over-expression in two of the lines, elav-GAL4(III)/UAS-lov20-mCherry; elav-GAL4(III)/UAS-lov22, shared defects in courtship (Figure 8-4). For elav-GAL4(III)/UAS-lov20-mCherry, the Courtship Index was determined to be 0.30, which is in a similar range to the lov mutants. The behavior in the courtship assay appeared to be very similar to the lov mutants. The males were inactive and would only court when the female was in close proximity. elav-GAL4(III)/UAS-lov22 males likewise had defects in courtship. Although the overall courtship of the males is high, the vigor

UAS-lov Line	Embryo phenotype	Adult phenotype	Courtship
UAS-lov4-mCherry	N/A	Reduced fertility in sib-mate:33% hatch rate	N/A
UAS-lov20-mCherry	N/A	i.)Reduced fertility in sib-mates: 32%,hatch rate ii.) very few adults from sib mates with fully unfolded wings.	N/A
UAS -lov22	N/A	i.)High rate of embryo lethality ii.) 20% of unhatched embryo unfertilized and 80% in advanced stages of development iii.)Sib-mate is lethal	N/A

Table 8-4: Results of Lov over-expression experiment with elav-GAL4 (II).

UAS-lov Line	Embryo phenotype	Adult phenotype	Courtship
UAS-lov4-mCherry	None	None	Normal
UAS-lov20-mCherry	None	1.6% flies with unfolded wings	CI is 0.30
UAS -lov22	None	0.84% males with unfolded wings	Normal CI but passive courtship

Table 8-5: Results of Lov over-expression experiment with elav-GAL4 (III).

with which they court is reduced. Many of the males spend a large fraction of the time sitting behind the female rather than actively courting her. This type of courtship was not observed in either parent line and is not observed in wild type flies.

The different effects of lov over-expression using the different elav-GAL4 lines suggest that the neurons of the normal elav pattern that do not express GAL4 with the elav-GAL4 (III) driver are important for larval and pupal development and the flies' ability to hatch and eclose. When lov is not overexpressed in those neurons, the effects of lov over-expression on adult behavior can be determined. It would appear that lov over-expression in the remaining neurons affects both the quantity and quality of male courtship. Thus elav-GAL4(III)/UAS-lov22, the flies had a normal Courtship Index but were very passive whereas elav-GAL4 (III)/UAS-lov20-mCherry males had a very low Courtship Index. This would suggest that ectopic expression of lov suppresses normal courtship.

#### lov over-expression with P0163

As shown in Chapter 4, Lov expresses in the PNS of the developing embryo. Therefore, the effect of lov over-expression in the PNS alone was examined. This was accomplished using the GAL4 line P0163 (Suster and Bate, 2002). P0163 expresses GAL4 in every neuron in the PNS allowing for the examination of lov in this component of the nervous system. The use of this driver will help determine whether the phenotypes observed with GAL4-elav over-expression are due to the over-expression of lov in the PNS or CNS. If the phenotypes previously observed also appear in the P0163 over-expression experiments, then those observed phenotypes are due to lov over-expression in the PNS.

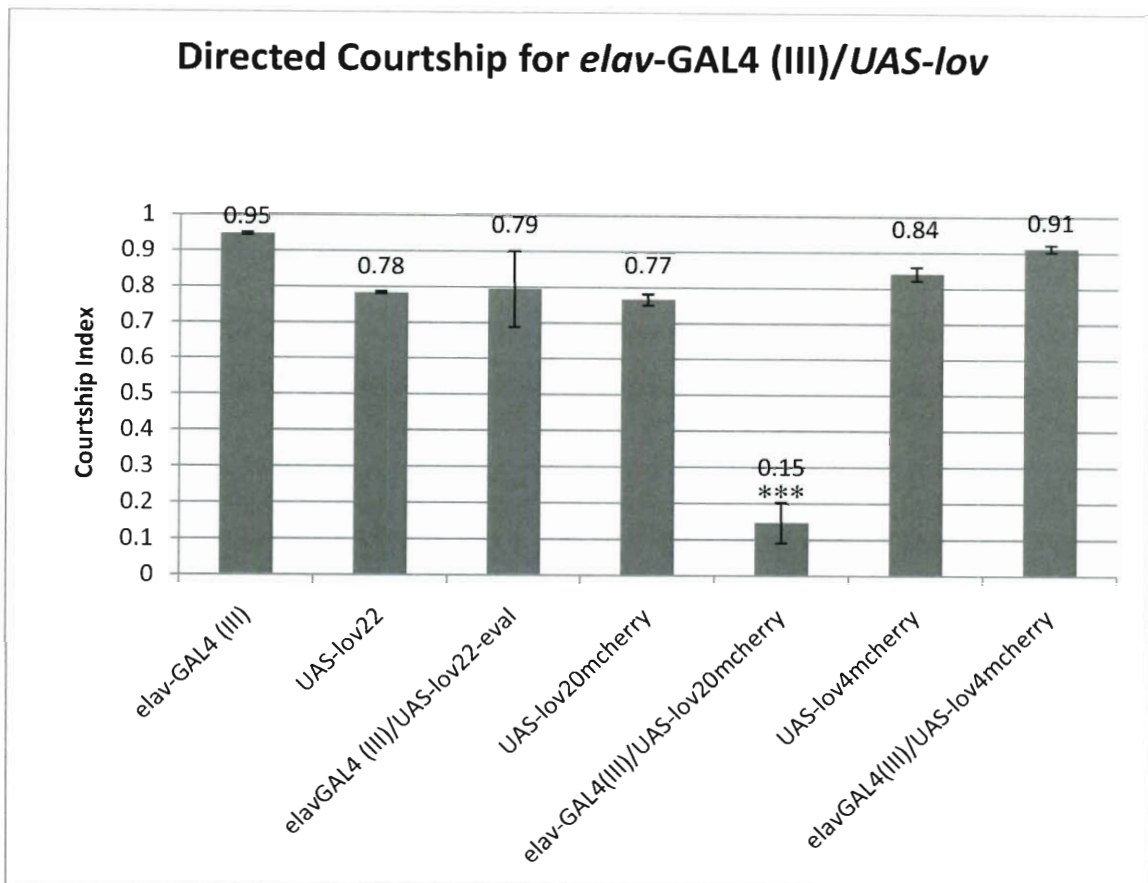


Figure 8-4: Directed Courtship Index (CI) for *elav-GAL4(III)/UAS-lov* males. For *elav-GAL4(III)/UAS-lov20-mCherry*, the Courtship Index is in a similar range to the *lov* mutants. While the overall Courtship Index for *elav-GAL4(III)/UAS-lov22* is high, the vigor with which the males court is reduced. Many of the males spend a large amount of time sitting behind the female rather than actively courting the female. A one-way ANOVA and T-tests between each *GAL4/UAS-lov* line and its parent lines were performed to determine statistical significance. (*elav-GAL4(III)* n=10; *elav-GAL4(III)/UAS-lov22*: p=0.21; *UAS-lov22* n=10; *elav-GAL4(III)/UAS-lov22* n=22, *elav-GAL4(III)/UAS-lov20-mCherry*: p≤ 0.001 *UAS-lov20-mCherry* n=9; *elav-GAL4(III)/UAS-lov20-mCherry* n=10, *elav-GAL4(III)/UAS-lov4-mCherry*: p= 0.03 *UAS-lov4-mCherry* n=10; *elav-GAL4(III)/UAS-lov4-mCherry* n=10)

As with the *elav*-GAL4 over-expression experiments, UAS-*lov4*-mCherry, UAS-*lov20*-mCherry, and UAS-*lov22* were all crossed to the P0163 GAL4 driver line. The resulting offspring were examined for defects in development and behavior.

When P0163/UAS-*lov4*-mCherry and P0163/UAS-*lov20*-mCherry were examined, there were no obvious phenotypes (Table 8-6) and males had normal courtship. In contrast, P0163/UAS-*lov22* survived larval life but showed a low adult eclosion rate of 8.3%. This is because many of the larvae die in first instar larvae. Additionally, the surviving adults from these experiments died much earlier than normal. 80 to 90% of males died before seven days of age, when males are normally tested for courtship defects. In contrast, none of P0163/UAS-*lov4*-mCherry and P0163/UAS-*lov20*-mCherry males died so early. Furthermore, when assayed, several of the males made little to no attempts to court females, resulting in a low Courtship Index of 0.47 (Figure 8-5). These results suggest that some of the effects observed in the *elav*-GAL4 over-expression experiments are due to over-expression in the PNS (Figures 8-4 & 5). When there is strong over-expression of *lov* in the PNS, the flies are unable to eclose and those that do eclose die sooner than expected. Therefore, the low eclosion rate observed in the *elav*-GAL4 over-expression experiments may be due the over-expression of *lov* in the PNS rather than the CNS. Additionally, strong expression leads to reduced courtship in several of the males, although this may be attributed to those flies being close to death.

#### *lov* over-expression with *atonal*-GAL4

As described in Chapter 4, during embryonic development, *Lov* expression is found in the PNS in each of the three sub-types of neurons. One of these subsets is the Chordotonal Organ neurons (Figures 4-10 & 11). In addition, 91Y was found to drive

UAS-lovLine	Embryo phenotype	Adult phenotype	Courtship
UAS-lov4-mCherry	None	Reduce fertility in sib-mates; 9% hatch rate	Normal
UAS-lov20-mCherry	None	Reduced fertility in sib-mates; 17% hatch rate	Normal
UAS-lov22	None	i.)Many larvae die as first instar ii.)Low eclosing rate (13/157 = 8.3%) iii.)80-90% of adults die in 7 days iv.)Reduced fertility in sib-mates-no eggs	CI is 0.47

Table 8-6: Results of Lov over-expression experiment with P0163 GAL4.

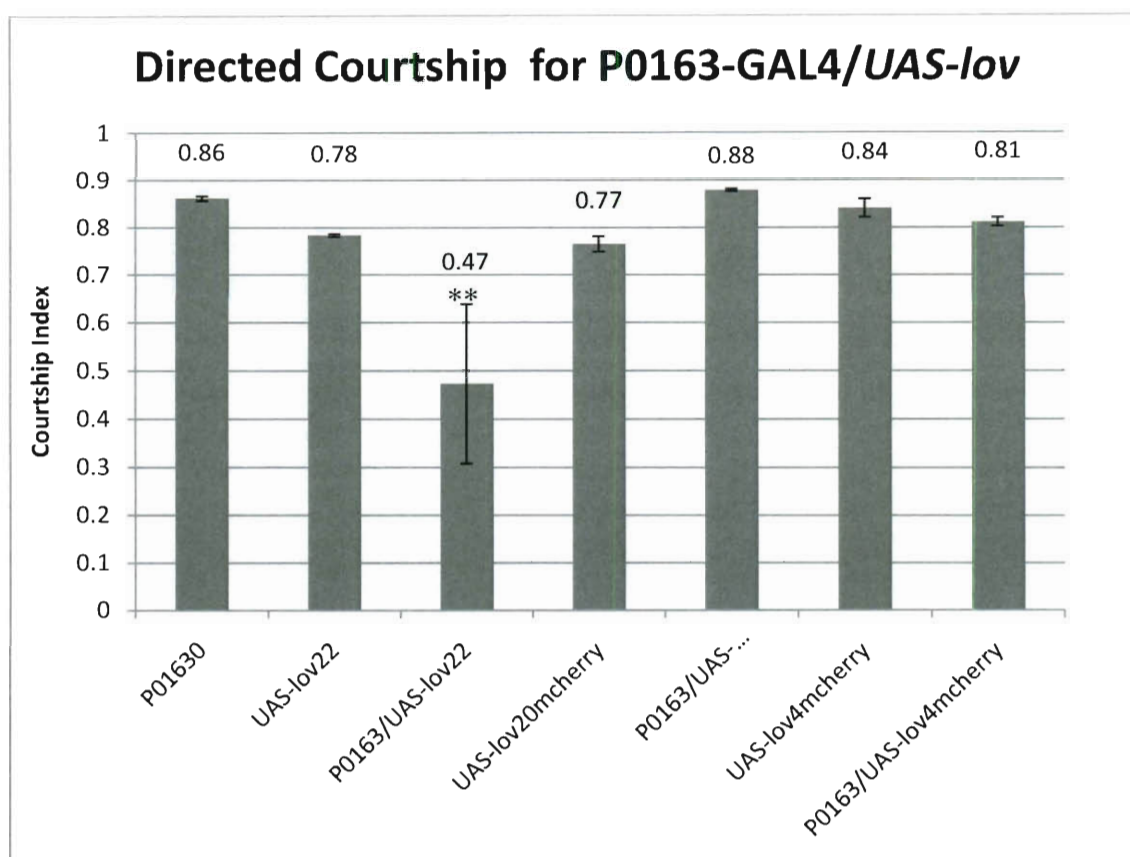


Figure 8-5: Directed Courtship index for P0163/UAS-lov. The CI for the parent P0163 GAL4 parent line is shown at the far left and the CI for the each UAS-lov parent line is next to the P0163 GAL4/UAS-lov line. For both P0163/UAS-lov4-mCherry and P0163/UAS-lov20-mCherry, males had normal courtship indices. For P0163/UAS-lov22, several of the males made little or no attempt to court females, resulting in a low courtship index of 0.47. A one-way ANOVA and T-tests between each GAL4/UAS-lov line and its parent lines were performed to determine statistical significance. (P0163 n=10; P0163/UAS-lov22: p=0.003; UAS-lov22 n=10; P0163/UAS-lov22 n=8, P0163/UAS-lov20-mCherry: p=0.02; UAS-lov20-mCherry n=9; P0163/UAS-lov20-mCherry n=10, P0163/UAS-lov4-mCherry: p= 0.60; UAS-lov4-mCherry n=10; P0163/UAS-lov4-mCherry n=9)



EYFP expression in the adult leg Chordotonal Organs (Figure 2-2). Therefore, Lov over-expression in the chordotonal organs was performed to examine what happens when lov is only overexpressed in chordotonal organs. It would also help to determine if the eclosion and courtship defects observed in P0163/UAS-lov22 are due to over-expression of lov in the chordotonal organs. An atonal-GAL4 insertion on the third chromosome was used for these studies. atonal is the proneural gene for the chordotonal organs; therefore, GAL4 will be expressed in every chordotonal neuron.

As can be seen from Table 8-7, over-expression of lov in the chordotonal organs has little effect. There are no obvious embryonic phenotypes in any of the lines. For all the lines tested, courtship is normal, with the Courtship Index being at or above wild type levels. For atonal-GAL4/UAS-lov22 and atonal-GAL4/UAS-lov20-mCherry, several of the males extended both wings during courtship. However this behavior was also observed in the parent UAS-lov lines. The only phenotype observed was some early death of atonal-GAL4/UAS-lov22 adults. By day 5 18% of adults were dead.

It would appear that while lov is normally expressed in a subset of chordotonal organs, ectopic expression of lov in the chordotonal organs does not have an effect on development and behavior. This would suggest that the courtship and eclosion defects observed in P0163/UAS-lov20-mCherry are due to lov over-expression in other peripheral neurons. Given that lov expression is also found in the external sensory cells, it may be the over-expression of lov in these cells that is leading to the observed defects in P0163/UAS-lov20-mCherry.

lov over-expression with fruM-GAL4

The final over-expression experiment performed used a fruM-GAL4 line. This

UAS-lov Line	Embryo phenotype	Adult phenotype	Courtship
UAS-lov4-mCherry	None	None	Normal
UAS-lov20-mCherry	None	None	Normal
UAS -lov14	None	None	Normal
UAS -lov22	None	18% lethality by Day 5	Not tested

Table 8-7: Results of Lov over-expression experiment with atonal-GAL4.

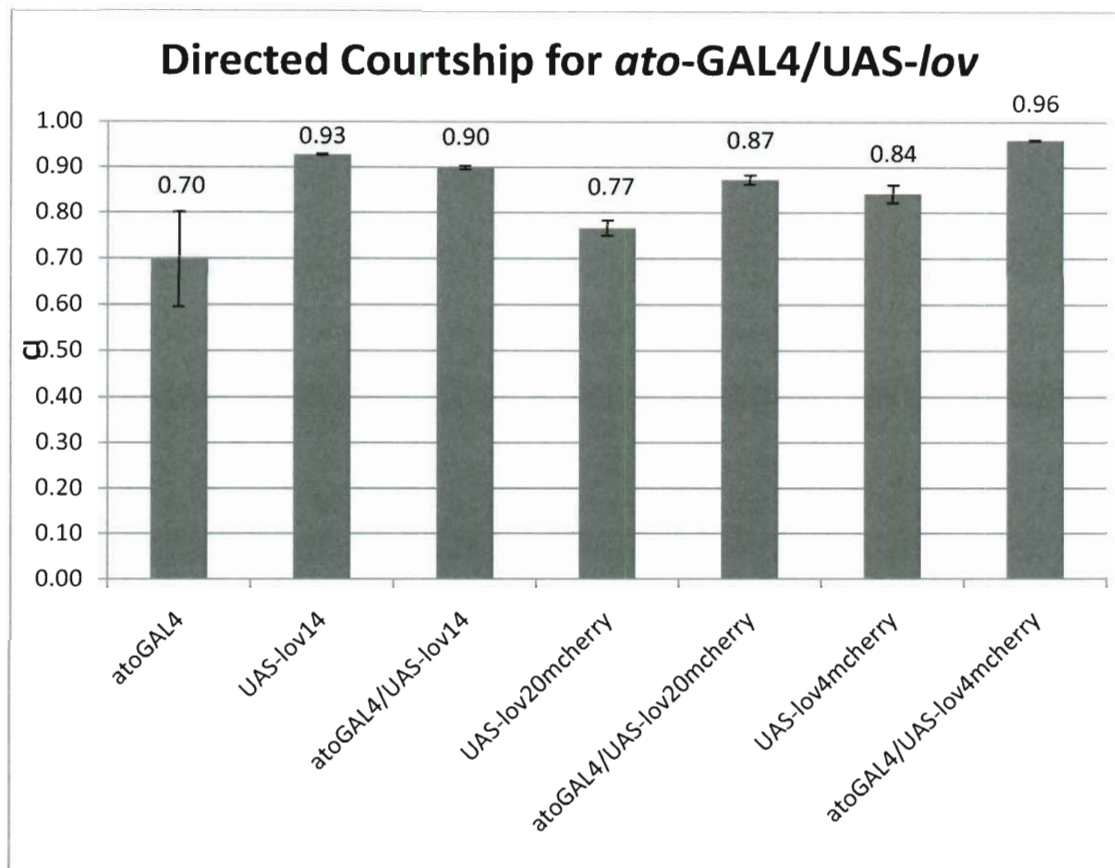


Figure 8-6: Directed courtship for the UAS-lov lines crossed to atonal-GAL4. For all three UAS-lov lines crossed to atonal-GAL4, the courtship was normal. A one-way ANOVA and T-tests between each GAL4/UAS-lov line and its parent lines were performed to determine statistical significance. ( ato-GAL4 n=10; ato-GAL4/UAS-lov14: p=0.02; UAS-lov14 n=10; ato-GAL4/UAS-lov14 n=10, ato-GAL4/UAS-lov20-mCherry: p=0.20; UAS-lov20-mCherry n=9; ato-GAL4/UAS-lov20-mCherry n=10; ato-GAL4/ UAS- lov4-mCherry: p=0.03; UAS-lov4-mCherry n=10; ato-GAL4/UAS-lov4-mCherry n=10)

line expresses GAL4 in the pattern of the male-specific form of Fruitless. By using this line, lov will be overexpressed in neurons which are known to be involved in courtship. As discussed earlier, one interpretation of the findings for the effects of some of the lov mutants on courtship is that there is a reduction of courtship when there is over-expression of lov. If this is the case, then the over-expression of lov in neurons known to be important in courtship could recapitulate the courtship defect.

When lov is expressed in fru expressing neurons, there is a reduction in courtship. For fruM-GAL4/UAS-lov4-mCherry, the Courtship Index is 0.49 (Table 8-8 and Figure 8-7). fruM-GAL4/UAS-lov20-mCherry has a Courtship Index of 0.14 and fruM-GAL4/UAS-lov22 has a Courtship Index of 0.30. These Courtship Indices are similar to the Courtship Indexes observed for the lov mutants. For all three lines, the reduction in Courtship Index is statistically significant when compared to the parent lines.

Various mutations to fru lead to male-male courtship. They also lead to the formation of courtship “chains” in which at least three males court in a line. Therefore, the possible formation of courtship chains when lov is over-expressed in the fru neurons was investigated. It was found that the expression of lov in these neurons does not lead to the formation of courtship chains (Table 8-8).

#### Summary for lov over-expression studies

The over-expression experiments using the various combinations of GAL4 drivers and UAS-lov demonstrate what happens when lov is over-expressed. Over-expression of lov can affect development, hatching, eclosion and courtship behavior. Given the finding that several of the lov mutants alter courtship, the alteration of courtship upon lov over-expression is particularly interesting. It supports the possibility that the reduction of

UAS-lov Line	Embryo phenotype	Adult phenotype	Courtship
UAS-lov4-mCherry	None	None	CI= 0.49
UAS-lov20-mCherry	None	None	CI=0.15
UAS -lov22	None	None	CI=0.30

Table 8-8: Results of Lov over-expression experiment with fruM-GAL4.

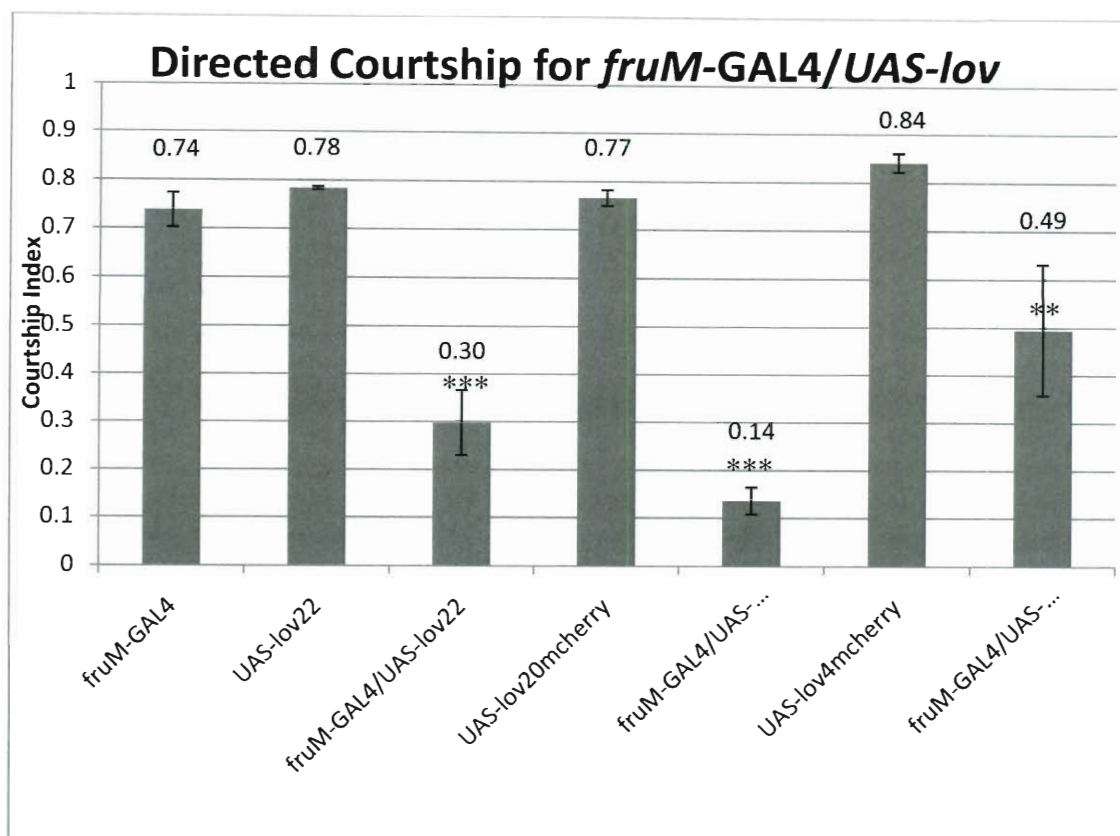


Figure 8-7: Directed courtship for the UAS-lov lines crossed to fruM-GAL4. When lov is expressed in fru expressing neurons, there is a reduction in courtship, suggesting that lov and fru may interact. A one-way ANOVA and T-tests between each GAL4/UAS-lov line and its parent lines were performed to determine statistical significance. ( fruM-GAL4 n=12; fruM-GAL4/UAS-lov22:  $p \leq 0.001$ ; UAS -lov22 n=10; fruM-GAL4 /UAS-lov22; n=9, fruM-GAL4/UAS-lov20-mCherry:  $p \leq 0.001$ ; UAS-lov20-mCherry n=10; fruM-GAL4/UAS-lov20-mCherry n=10, fruM-GAL4/UAS-lov4-mCherry:  $p=0.01$ ; UAS-lov4-mCherry n=10; fruM-GAL4/UAS-lov4-mCherry n=10)

courtship in some *lov* mutants may reflect *lov* over-expression. *Lov* over-expression alters courtship when it is expressed in *FruM* expressing neurons. This further supports *lov*'s role in courtship and suggest a possible interaction of *Fru* and *Lov*. As previously discussed in the Introduction, both proteins contain a BTB domain that can dimerize with a BTB domain in other proteins. Therefore, it is possible that *Fru* and *Lov* are interacting through the BTB domain to regulate courtship behavior.

When the effects of *lov* over-expression driven by the P0163 *GAL4* driver are compared to the effects of *lov* over-expression driven by the *atonal-GAL4* driver clear differences are detected. The over-expression *lov* with P0163 results in a decrease in the longevity of the resulting progeny and a reduction in courtship probably because the males in question were close to death. Over-expression of *lov* in the Chordotonal Organs through the use of *atonal-GAL4* did not result in the same decrease in longevity and courtship. Therefore, the observed decrease in longevity in P0163 is not due exclusively to the over-expression of *lov* in the Chordotonal Organs.

Finally, when the effects of *lov* over-expression on courtship are compared for the *elav-GAL4* (III) driver versus *fruM-GAL4* driver, the effect on courtship is more severe when *lov* is over-expressed using the *fruM-GAL4* driver. There are two possible explanations for this. First, it is possible that the *fruM-GAL4* driver is a stronger driver line than *elav-GAL4* (III), resulting in higher expression levels of *Lov* and therefore stronger behavioral effects. Second, the *elav-GAL4* may drive over-expression of *lov* in *FruM*-expressing neurons and other neurons. It is possible that over-expression in additional neurons modifies the effects of *lov* over-expression in the *FruM* neurons.

## Under-expression Experiments using Valium-lovRNAi

While the over-expression experiments with *lov* demonstrate potential roles of *lov* and help to provide further evidence for a role of *lov* in courtship, some of the effects detected may reflect *Lov* acting in cells outside its normal expression pattern. To examine the function of *lov* in its normal expression pattern, under-expression of *Lov* using a Valium-lovRNAi transgenic line was examined. This line was generated by the Transgenic RNAi Project (TRiP) at Harvard Medical (FlyRNAi.org) and has several advantages over other RNAi lines. First, the eye color vermilion was used in place of the mini-white gene that is traditionally found in pUAST vectors because it allows for better gene dosage than white. Additionally, the RNAi vector contains ten UAS binding site as opposed to the typical five binding sites which allows for improved expression of the transgene. Finally, the VALIUM vectors were based on the phiC31 targeted integration method (Groth et al., 2004) which allows for the selection of the location of the transgene. In this way, the transgene is inserted into a location that produces maximum expression of the transgene under control of GAL4 drivers.

Expression of lovRNAi transgene was first driven by an *elav-GAL4(X)* line. This line also contains UAS-dcr2, which is a dicer transgene. dicer is a component of RNA interference pathway that cleaves double-stranded RNA (Dietzl et al., 2007). This will help to further reduce the expression of *lov*. The *elav-GAL4(X)/UAS-dcr* drives expression of lovRNAi in every neuron. However, *lov* expression will only be reduced in the cells that normally express *lov*.

As with the over-expression experiments, the effects of under-expression using the GAL4 drivers were assessed in progeny from a cross of a line carrying the GAL4



driver with a line homozygous for a UAS-lov construct. Progeny were analysed as for the over-expression experiments (see Material and Methods)

As can be seen from Table 8-9, reduction of lov during embryogenesis does not appear to affect development as there are no embryonic phenotypes. Additionally, normal adults are produced. However, it appears that reduced lov under-expression in adults affects fertility. The sib-mated adult males or females from this experiment produce embryos that die in early development. The elav-GAL4/UAS-dcr/Valium-lovRNAi adults were individually mated to w<sup>1118</sup> (a control line) partners to determine which sex was responsible for the infertility. Surprisingly, the elav-GAL4/UAS-dcr/Valium-lovRNAi males appear to be responsible for this effect. elav-GAL4/UAS-dcr/Valium-lovRNAi females produced viable offspring, whereas w<sup>1118</sup> females, when mated to elav-GAL4/UAS-dcr/Valium-lovRNAi males, laid eggs (suggesting they had been mated) but none of the eggs hatched. This is an interesting result as males contribute little to the developing embryo other than DNA and centrosomes. Further studies are needed to understand this result.

When the ability of elav-GAL4/UAS-dcr/Valium-lovRNAi males to court was tested, there was a significant reduction in courtship (Figure 8-8). The courtship index is 0.21, which is significantly lower either parent line. Thus reduced levels of lov lead to defects in courtship.

Next, Valium-lovRNAi expression was driven using the P0163 GAL4 driver described above. In using this line, the contribution of lov expression in PNS for normal courtship behavior can be examined. Again, while the P0163 line will drive expression of lovRNAi in every neuron in the PNS, lov expression will only be reduced in neurons that

express *lov*. The same set of experiments were performed for these lines as was for the *elav-GAL4/UAS-dcr/Valium-lovRNAi* lines.

A reduction of *lov* in the PNS does not appear to affect development. No obvious phenotypes were visible in either embryos or adults. Furthermore, when sib-mating was performed using the adult progeny, they were able to produce offspring (Table 8-9). Therefore, a reduction of *lov* in the PNS does not cause defects in fertility. Finally, a reduction of *lov* expression in the PNS does not result abnormal courtship behavior. This suggests that *lov* expression in the PNS is not required for normal courtship behavior (Figure 8-8).

Finally, *Valium-lovRNAi* expression was driven using the *fruM-GAL4* described above. This examined the contribution of *lov* expression in neurons known to be involved in courtship for normal courtship. If *lov* is expressed in these neurons, alterations in courtship behavior should be observed. The same set of experiments was performed for these lines as was for the *elav-GAL4/UAS-dcr/Valium-lovRNAi*.

A reduction of *lov* expression in the *fru* expressing neurons did not cause defects in development as there were no obvious embryonic or adult phenotypes. Furthermore sib-mated adults from these crosses produced viable offspring demonstrating that they are fertile. However, when courtship was examined, there was a significant reduction in courtship as compared to either parent line. However, the reduction is not as great as the reduction when *lov* expression is reduced using the *elav-GAL4(X)/UAS-dcr* driver. This suggests two possibilities. First, the reduction of *lov* in the *fruM-GAL4* expressing neurons is not as complete as the *elav-GAL4(X)/UAS-dcr* neurons due to the presence of the *dcr* gene, which is part of the RNAi pathway and results in a greater reduction in *lov*

GAL4 Line	Embryo phenotype	Adult phenotype	Courtship
elav-GAL4/UAS-dcr(X)	None	Sibmate = Embryo lethal Most embryos die in very early development. Paternal fertility problem	CI=0.21
P0163	None	Sibmate ok	Normal
fruM-GAL4	None	Sibmate ok	CI=0.54

Table 8-9: Results of RNAi experiments with Valium-lovRNAi.

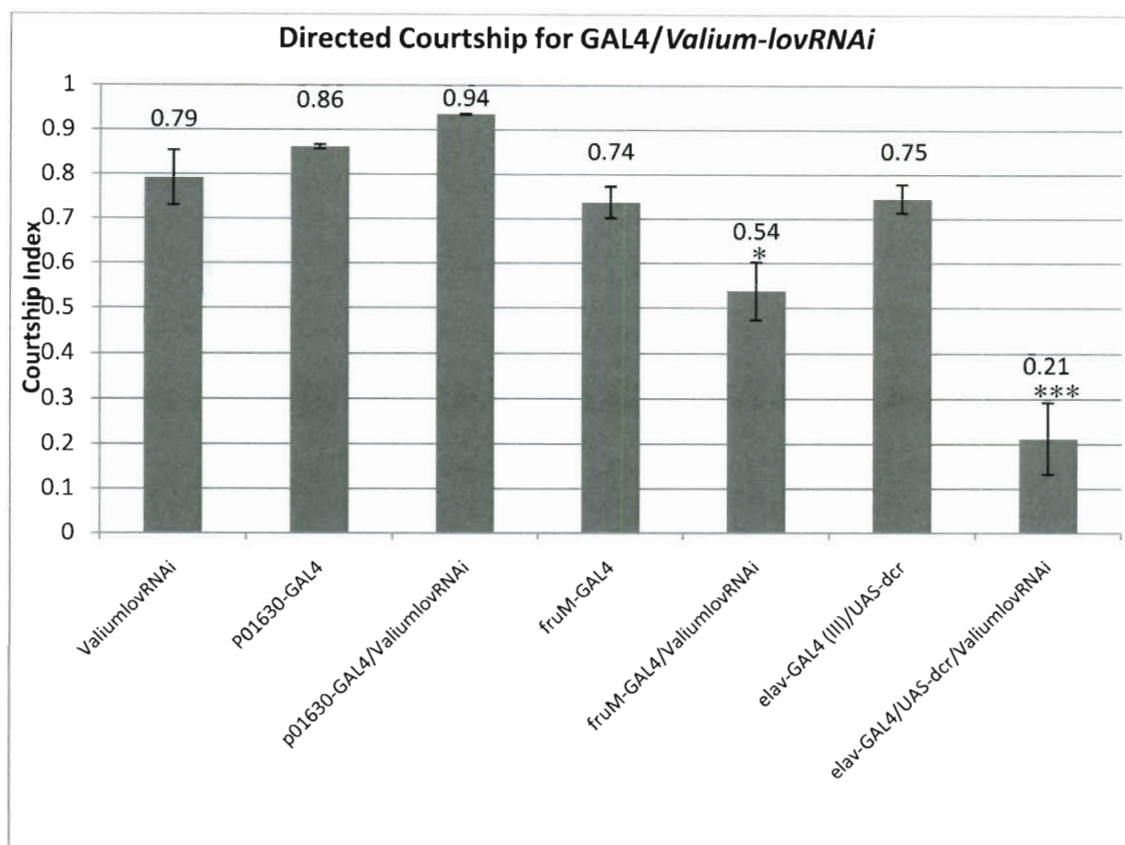


Figure 8-8: Directed courtship for the GAL4 lines crossed to Valium-lovRNAi. When the Valium-lovRNAi was crossed to either elav-GAL4(X) /UAS-dcr or fruM-GAL4, there was a significant reduction in courtship. A one-way ANOVA and T-tests between each GAL4/Valium-lovRNAi line and its parent lines were performed to determine statistical significance. (Valium-lovRNAi  $n=10$ ; P0163/Valium-lovRNAi:  $p=0.12$ ; P0163-GAL4  $n=10$ , P0163/Valium-lovRNAi  $n=10$ ; fruM-GAL4/Valium-lovRNAi:  $p=0.05$ ; fruM-GAL4  $n=12$ , fruM-GAL4/Valium-lovRNAi  $n=10$  elav-GAL4(X)//UAS-dcr/Valium-lovRNAi  $p\leq 0.001$  elav-GAL4/UAS-dcr(X)  $n=10$ , elav-GAL4/UAS-dcr(X)/Valium-lovRNAi  $n=10$ )

expression. Second, lov may regulate courtship through the use of additional neurons beyond the fru expressing neurons.

What do the lov mis-expression and lov-RNAi experiments tell us about the function of lov?

The UAS-lov and UAS-lov-mCherry over-expression experiments confirm that the courtship defect observed in the lov mutants may be due to the over-expression of lov. When UAS-lov and UAS-lov-mCherry are expressed in FruM expressing neurons, there is a reduction in courtship. This reduction in courtship helps confirm that lov does have a role in courtship. It also suggests that Lov and Fru may interact, possibly through their BTB domains.

The reduction of lov through the use of the fruM-GAL4 and elav GAL4(X) demonstrates that lov expression is required for normal courtship. The greater reduction in courtship when elav GAL4(X) driver is used could be due to two factors. First the presence of the UAS-dcr in this line reduces the level of lov more effectively and therefore lov expression may be lower in the elav GAL4(X) as compared to the fruM-GAL4 line, resulting in the greater reduction in courtship. Second, lov may function to regulate courtship through neurons outside of FruM expressing neurons. Reduction of lov expression using the P0163-GAL4 driver, which expresses in the PNS, suggest that lov expression in the PNS is not involved in regulating courtship behavior. Therefore, the alterations in other behaviors discussed in Chapter 7 may be due to defects in processing sensory input rather than receiving sensory input. Furthermore, lov may act in the CNS to regulate courtship behavior and examination of lov expression in the CNS might identify new locations important in courtship behavior. The reduction of courtship when lov is either over or under-expressed suggests that levels of lov must be tightly regulated for

normal courtship to occur. Likewise, tight regulation of lov throughout the nervous system is required for normal development.

## Materials and Methods

### Generation of the lov-pUAST construct

The first transgenic construct generated was lov-pUAST construct. To generate this construct, the coding region of lov from lov cDNA G08221, which contains the entire protein coding region, was amplified using PCR using the following primers: pUAST-upstream 5'GTTGAATTCCTGGATACGAGAATTGAAGCACGC 3' which added an Eco R I restriction site and pUAST-downstream 5' GTTAGATCTGTTTCAATCATGCCCGGTC 3' which added a Xho I restriction site. The resulting PCR product was inserted into the pUAST vector using standard cloning techniques between the EcoR I and Xho I restriction sites. The vector was sequenced to confirm that there were no alterations in the sequence. The UAS-lov construct was then prepared for injection using Qiagen's HiSpeed Midi Plasmid Purification Kit following the enclosed protocol. The construct was then sent off to GenetiVision (Houston, TX) for injection into embryos.

### Generation of the lov-mCherry-pUAST construct

To generate this construct, the coding region of lov was amplified with PCR using the lov cDNA G08221 using the following primers: pUAST-upstream 5'GTTGAATTCCTGGATACGAGAATTGAAGCACGC3', which adds Eco R I restriction site and lov-mCherry-pUAST downstream 5'GTTAGATCTAAGTATCAATAATGCCCGGTC3', which adds a Bgl II restriction site and also removed the stop codon to allow for translation of mCherry and add a linker sequence. Likewise, the coding region of mcherry was amplified with PCR using the mcherry vector (Clontech) with the following primers: mCherry upstream primer 5'

CTCGCCCTTGCTCACCATAGCAGATC3', which adds a Bgl II restriction site and an alanine to the linker and mCherry downstream primer 5'GCGGCCGCCTACTTGTACAGCTCGTCC3', which adds a Not I restriction site. lov-mCherry-pUAST downstream and mCherry upstream primers created a linker of leu, lle and ala (leu leu lle leu ala) so that both proteins could fold properly without interference from the other protein. lov-mCherry-pUAST downstream primer modified the stop codon to a leu as well as modify the immediate downstream sequence to leu lle leu to become part of the linker. mCherry upstream primer contributed an alanine to the linker. The resulting PCR products were inserted into the pUAST vector between the Bgl II and Not I restriction sites using standard cloning techniques. The vector was sequenced to confirm that there were no alterations in the sequence. The UAS-lov-mCherry construct was then prepared for injection using Qiagen's HiSpeed Midi Plasmid Purification Kit following the enclosed protocol. The purified construct was then sent off to GenetiVision (Houston, TX) for injection into embryos. The resulting larvae were then returned for screening and generation of transgenic stocks.

#### Generation of transgenic lines

The injected larvae were allowed to mature to adult flies. The resulting males and females were then crossed to a balancer line and a series of crosses were performed to isolate individual insertions (Figure 8-1). The locations of the insertions were identified through the use of the balancers used to generate the transgenic lines (Figure 8-2).

#### Description of the over- and under-expression experiments

For each over and under-expression experiment, adults from the lov transgenic line and adults from a GAL4 line were placed together for mating in agar grape plates. After being



placed one day together, the animals were transferred into a new plate. One hundred eggs laid on the first agar grape plate were transferred to a new plate within 24 hours. After another 24 hour wait, the number of eggs that hatched were tallied. Those eggs which did not hatch were examined to determine if they were fertilized or unfertilized. The fertilized eggs were examined to determine their level of development. The embryos that did hatch were then followed throughout development to determine if there is a lethality defect and any other phenotype due to over-expression of *lov*. Any adults that eclosed were tested for courtship and/or their ability to produce offspring, depending on the location of the *GAL4* insertion.

#### Courtship Assays

Naïve males were collected and allowed to age seven days. They were then placed in courtship wheels with 3-5 day old Control females (Canton S) virgin females and recorded for 10 minutes (O'Dell, 2003). The amount of time the males courted the female was determined and the Courtship Index was calculate as the fraction of the time the male spends courting the female (Villella et al, 1997).

#### Statistical Analysis

For the courtship assays, a one-way ANOVA was used to compared each combination of *GAL4/UAS-lov* or *GAL4/VALIUM-lovRNAi* with both of its parent lines. If the diffences in the Courtship Indices were found to be statistically significant, a T-test was used to compare that *GAL4/UAS-lov* or *GAL4/VALIUM-lovRNAi* with each of its parent lines. An alpha of 0.05 was used for all statistics. The number of males (n) tested and the p-value is listed in each figure legend. Statistical significance is indicated in each figure in the following way:  $*=p \leq 0.05$ ;  $**p \leq 0.01$ ;  $***p \leq 0.001$ .

## CHAPTER 9: IDENTIFICATION OF lov INTERACTION PARTNERS

One way to gain insight into the role of lov in *Drosophila* is to identify its protein interacting partners. Based on sequence data, it is believed that lov is a transcription factor. Furthermore, lov encodes a protein with a BTB domain, which is known to be a dimerization domain. Like Lov, other known or putative transcription factors containing this domain are expressed neurons and are believed to be important in various behaviors. Therefore, it is likely that Lov may interact with this group of proteins.

One such protein is Fru. As previously mentioned, fru is absolutely required for normal *Drosophila* courtship. A male-specific isoform, which is generated from male specific transcripts, is responsible for proper courtship. Similarly, lov may have a male specific transcript and this transcript is required for normal courtship. Given these similarities and that the fact that lov and fru both encode BTB domain proteins, the possibility that Lov and Fru may interact and that this interaction might be required for proper courtship was investigated.

To determine what Lov interacts with, identification of proteins through co-immunoprecipitation with Lov was used. Two columns were created in which the Lov antibody was cross-linked to resin and then used to extract Lov and anything else bound to Lov. Mass spectrometry was then used to identify the proteins that were pulled out. Generation of a Lov antibody column by the Seize technique

An initial column was generated through the use of the Pierce Seize Primary Immunoprecipitation Kit. This kit couples the Lov antibody directly to its AminoLink Coupling Gel, a resin that immobilizes the antibody. The surface of the resin is covered with aldehyde groups that react with lysine epsilon-amines on the surface of the antibody

to form reversible Schiff-base bonds. These bonds are reduced and made irreversible to secondary amine bonds by the addition of cyanoborohydride. This creates a reusable column and prevents the antibody from eluting with the target protein.

To make this column, the concentration of the affinity-purified Lov antibody (described in Chapter 4) was first determined using a Bradford assay. Next, a buffer exchange was performed to remove any primary amines that may interfere with the antibody binding to the resin. Once the antibody was in the correct buffer, the antibody was combined with the resin and incubated overnight at 4° C. A reducing agent, Sodium Cyanoborohydride, was then added to permanently link the antibody to the resin. Next, a quenching buffer was added to stop the immobilization reaction. Finally, a series of washes was performed to remove any excess antibody.

To test whether the column could bind Lov, the column was tested with the Lov fusion protein that was used to generate the antibody. The fusion protein consists of a small antigenic fragment (218 amino acids) unique to Lov and a GST tag that was used for purification of the fusion protein (for more detailed explanation see Chapter 4). The Lov fusion protein was incubated on the antibody column overnight and then eluted off the column using a buffer provided with the kit. The elutions were then run on 12.5% acrylamide gel. As can be seen in Figure 9-1, the column did in fact pull out the Lov fusion protein, showing that the column works. However, the amount of peptide that was eluted is small compared to the amount loaded.

#### Generation of Lov Antibody Protein A Column

Because of the amount of Lov peptide that eluted with the Seize column was small, a second method was used to make an immunoprecipitation column to see if a

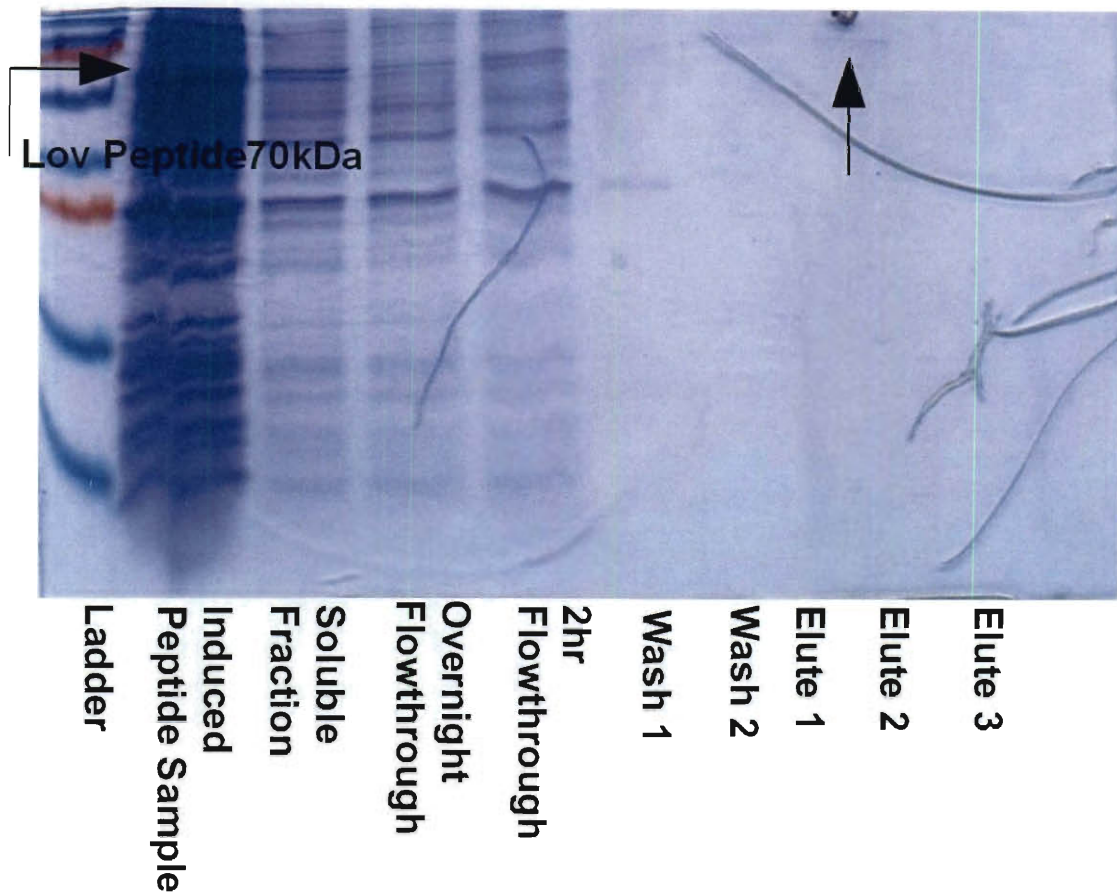


Figure 9-1: Gel of the Seize Column Pull-down of the Lov Peptide. The Seize Column was able to pull out Lov fusion protein from the bacterial lysates. The Lov fusion protein is the 70 kDa band highlight by an arrow in Elute 2 (it appears slightly higher than 70 kDa due to the way the gel ran). The yield off the column was low when compared to what was loaded (soluble fraction).

better yield could be achieved. This time a Protein A Antibody affinity column was made.

As before, the antibody underwent a buffer exchange to remove any primary amines. The antibody was then combined with the Protein A resin beads and allowed to incubate for one hour. A reducing agent, dimethylpimelimidate was added to covalently link Protein A and the Lov antibody. Finally, the cross linking reaction was stopped by two washes with ethanolamine.

Again, the column was tested to determine whether the column was effective at binding Lov or not by running the Lov fusion protein over the column. The fusion protein was incubated on the antibody column overnight and then eluted off the column using the same buffer that was used with the seize column. The elutions were then run on 12.5% acrylamide gel. As can be seen from Figure 9-2, the Lov fusion protein binds and elutes from the Protein A column more effectively than from the Seize column. Therefore, for the immunoprecipitation experiments, the Protein A column was used.

#### Co-immunoprecipitation of Lov and interaction partners from head extracts using the Protein A column

Once the column for the immunoprecipitation was generated and shown to be able to bind the Lov fusion protein, the immunoprecipitation experiment was carried out using adult heads. Adult heads were chosen as the tissue to be tested for two reasons. First, heads are believed to have among the highest Lov expression in the animal (Flyatlas.org). Second, many of the behavioral defects observed in the various lov mutants are believed to be due to changes in the expression in the CNS. Therefore, determining what Lov is interacting with in the CNS will provide more insight into the role of lov with respect to behavior.

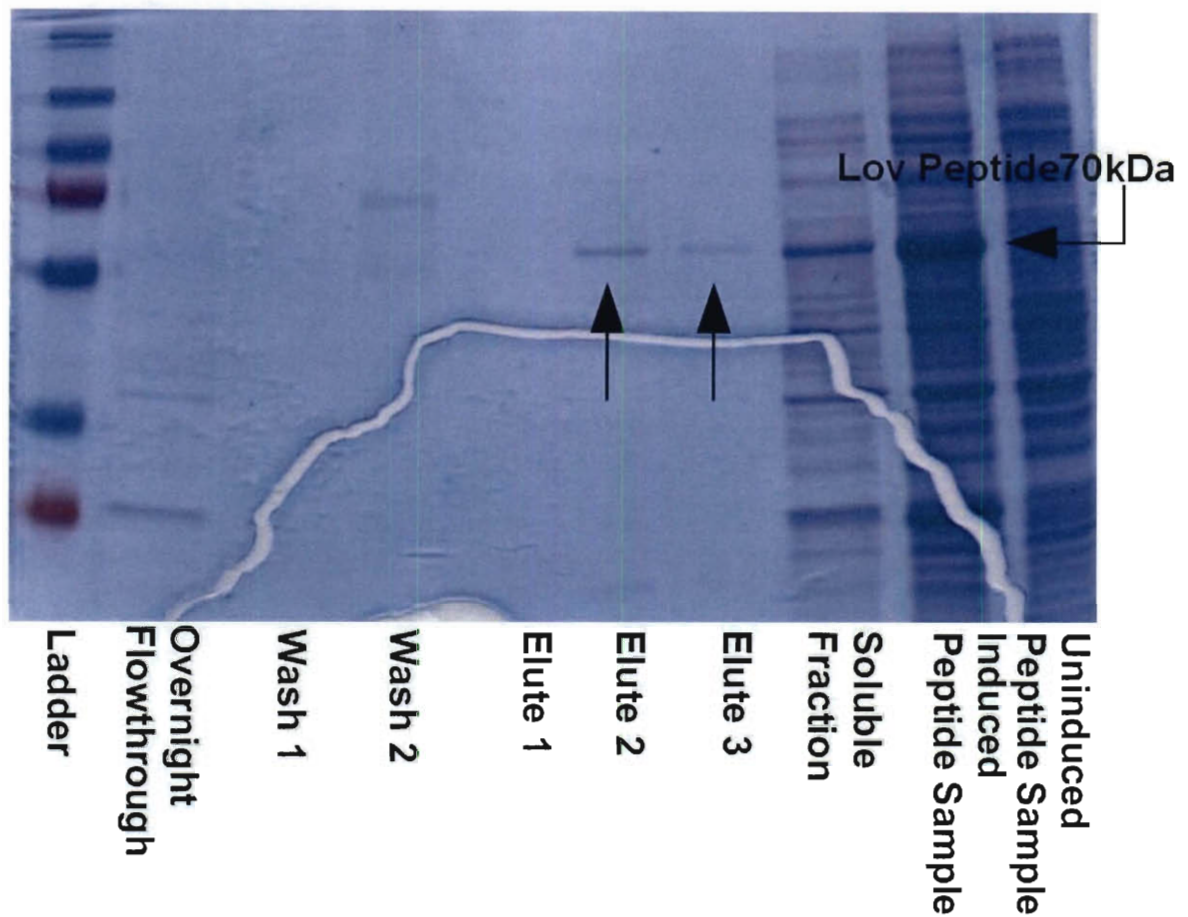


Figure 9-2: Assay of the Protein A Column for binding of the Lov Peptide. The Protein A Column was also able to bind Lov from the bacterial lysates. It is the 70 kDa band indicated by an arrow in Elute 2 and 3. The yield off the column was better than with the Seize Column.

For the co-immunoprecipitation, 1000 heads from both newly eclosed males and female were collected. Then, three different salt solutions, 50, 200, and 400 mM, were used to try to extract Lov and its interacting partners from the heads. The heads were first homogenized in the 50 mM salt solution. The supernatant was drawn off and the 200 mM salt solution was added and the heads were re-homogenized. The second supernatant was removed and a third extraction was performed with the 400 mM salt solution. This preparation of head extracts was performed twice. The plan was to run each salt extract over the column separately. However, in the first preparation, the 50mM and 200mM extracts for both males and females were accidentally combined before placing on the column. Therefore, in the first experiment, two samples (50/200mM combined sample and 400mM sample) were examined. Immunoblots were used for each experiment to determine which extracts contained the most Lov. Both extracts in the first experiment and all three extracts in the second experiment contained similar amounts of Lov. Therefore all the extracts from both males and females for both experiments were run over the column.

The extracts were incubated on the antibody column overnight and then eluted off the column as performed for the Lov fusion protein. The elutions were then run on 12.5% acrylamide gel and immunoblotted with the Lov antibody to identify the Lov protein. In the first preparation, for both male and female heads, under each salt condition and in each elution, three cross reacting bands at 70, 55, and 35 kDa were seen. Figure 9-3 shows the elutes for males and females from the 400mM salt extracts. As described in Chapter 4, the Lov antibody detects these same three bands in lysates of whole tissues. These three bands may reflect the major fragments of Lov present in tissues. For all three

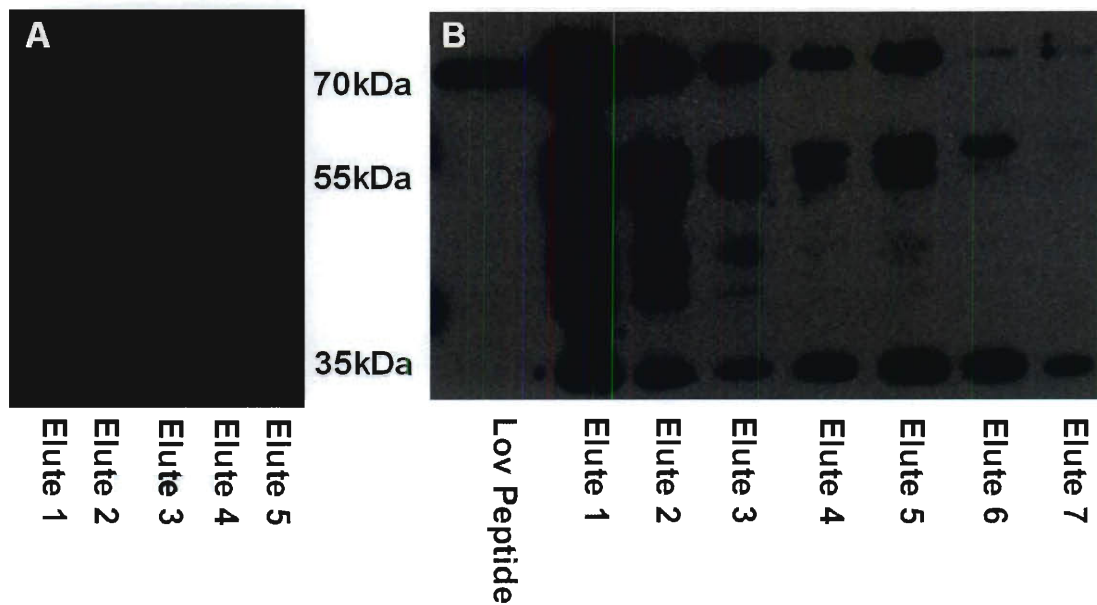


Figure 9-3: Immunoblots for male and female head 400 mM salt extracts (First Preparation). For both male (A) and female (B) heads, the Protein A column to extract the Lov antibody detects three bands at 70, 55, and 35 kDa. The antibody detects these same three bands in lysates of whole tissues and these bands may reflect the major fragments of Lov present in tissues. Similar results were observed for the 50/200 mM combined sample (Data not shown).



conditions, the majority of Lov was found in the first two elutions. The second preparation, in which the three salt conditions were passed over the column separately, yielded similar results. Again, when the elutions were run on a gel and probed for Lov with the Lov antibody, the three bands at 70, 55, and 35 kDa were present (Figure 9-4). Like the first preparation, the majority of Lov was found in the first two elutions. Based on the results from the two immunoblots, it would appear that the Protein A column is able to extract Lov.

The elutes from the Protein A column were too dilute to detect protein bands by Coomassie Blue staining. However, once it was determined that the Protein A column could extract Lov protein fragments, trichloroacetic acid (TCA) precipitation was used to concentrate the relevant elution fractions so that staining could be used to identify any bands which could potentially be Lov interacting partners. To accomplish this, an equal volume of 10% TCA was added to the elutions followed by incubation for up to two hours on ice. The samples were then centrifuged to pellet the precipitated protein. An equivalent volume of 5% TCA was then added and the samples were centrifuged a second time. Any contaminants that remained were then washed away with two washes with 95% ethanol and two washes with acetone. The samples were allowed to dry and SDS sample buffer was added.

#### Analysis of proteins bound to the Protein A column

The elutes from the 400 mM salt solution of both male and female heads from the first preparation were the first samples to undergo TCA precipitation and analysis. When these samples were run on an acrylamide gel and stained with Coomassie, several interesting bands appeared. First, in both the male and female sample, there is a band at

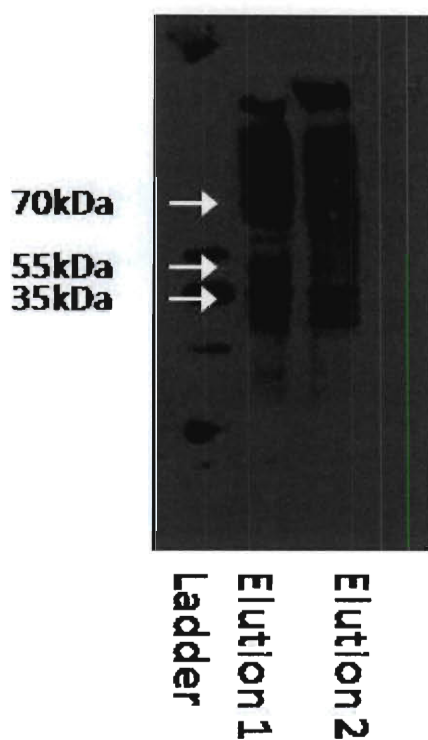


Figure 9-4: Immunoblots for male head 200 mM salt extracts (Second Preparation).

The Protein A column extracts that the Lov antibody cross reacts with are the same bands observed in whole tissue lysates at 70, 55, and 35 kDa. Similar bands were seen in the other extracts (data not shown).

250 kDa that is very strong and may represent a dimerization product of Lov.

Additionally, there is a band at 130 kDa, which is close to the predicted size of Fru. This band appears in immunoblots of heads when probed with an anti-Fru antibody (Figure 9-5). The presence of this band would suggest that Fru may be brought down with Lov. Next there are bands at 70, 55, and 30 kDa. These bands are the same size as the bands detected when whole heads are solubilized and probed with the Lov antibody. Finally, there is a band at 45 kDa which may be part of the Lov antibody. In previous co-immunoprecipitation experiments performed in the Beckingham Lab, this band was observed and identified as a degradation product of the heavy chain of the antibody (Beckingham, unpublished results).

Next, the first two elutions from the 50/200 mM salt solution from both male and female heads from the first preparation were concentrated and analyzed. When these samples were run on an acrylamide gel, only two bands appeared, both of which shared with the 400 mM elutions. There was a band at 250 kDa that again was strong relative to the other bands and a band at 45 kDa, which may be may be part of the Lov antibody.

No further samples were obtained from this first head extraction because significant protein loss occurred when the remaining elutes were TCA precipitated.

The 250 kDa, 60 kDa, 45 kDa, and 30 kDa bands found in the 400 mM and 50/200 mM extracts were selected to undergo mass spectrometry to determine their identity. The same size bands were combined from the analyses gels to provide maximum amount of sample for the mass spectrometry. To prepare the samples for mass spectrometry, the gels underwent three washes with 0.2  $\mu$  filtered water. The bands were then sliced from the gel, placed in eppendorf tubes, and allowed to dry.

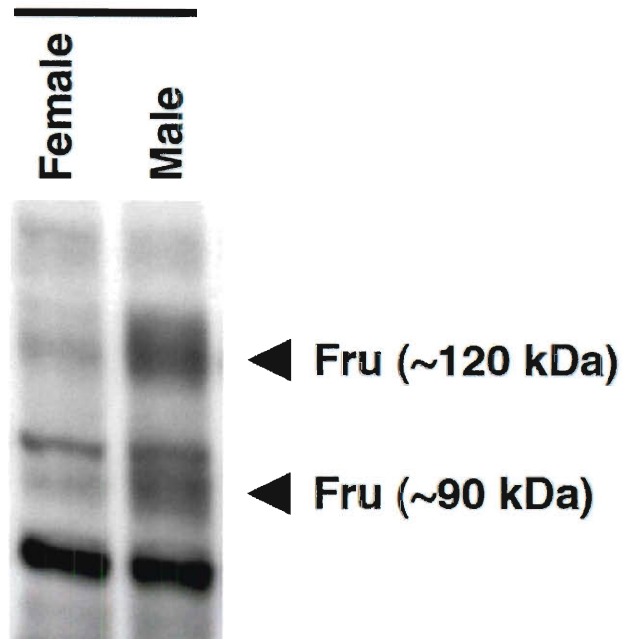
**Adult head extract**

Figure 9-5: Immunoblot of Head Tissue with Anti-Fru (male specific). In the male heads, there are two bands that cross react with an antibody against the male specific isoform of Fru (Gukuta Toba, personal communication). A band of similar weight to the larger of the two bands (120 kDa) was observed in the Coomassie gels from the co-immunoprecipitation with Lov, suggesting that Lov and Fru may be interacting

For the second head extraction experiment, TCA precipitation was performed for the first two elutions from the column for each salt condition since those two elutions contained most of the protein eluted from the column upon gel analysis. The female 50 mM preparation was found to have the most bands. Many of the same bands appeared in this second preparation as in the first preparation including the 250 kDa, 60 kDa, 45 kDa, and 30 kDa bands that were sent for mass spectrometry. Additional bands at 110 kDa, 100 kDa, 70 kDa, 40 kDa, 20 kDa, 17 kDa, 15 kDa were present in the second preparation that were not present in the first preparation. The bands at 70 kDa, 55 kDa, 45 kDa, and 40 kDa were chosen for mass spectrometry and prepared as described above.

#### Results from Mass Spectrometry

The first round of mass spectrometry yielded no information on any potential Lov interacting partners. Rather the results show contamination of two types. The first was keratin contamination, which is a common contaminant from human skin. Muscle tissue protein contaminants were the second type of contamination. The proteins identified besides keratin were Myosin, Actin, and Troponin. The identification of muscle specific protein does not fit with what is known about Lov. Lov is believed to be a transcription factor and immunolocalization has shown it localizes in the nucleus. Therefore it is unlikely that Lov would be interacting with these proteins. For this first round of co-immunoprecipitation, high salt extracts (200 and 400 mM) were being examined. High salt conditions solubilize muscle proteins presumably resulting in this contamination with muscle proteins.

For the second round of mass spectrometry, muscle contamination was avoided by focusing on the 50 mM and 200 mM salt solution samples. Once again there was keratin

contamination. However, other proteins were identified in the column elutes. These were: Apolipoproteins precursor, Larval serum protein 1 gamma chain precursor, ATP synthase subunit beta, mitochondrial precursor, ATP synthase subunit alpha, mitochondrial precursor, Actin, and Fructose-bisphosphate aldolase. None of these proteins have been identified as having a role in courtship. Rather, most of the proteins identified have a role in energy production. Larval serum protein 1 has been identified as contaminant in other mass spectrometry experiments (Yang, Sampson, Krause, 2006).

Neither round of mass spectrometry identified Lov as one of the proteins pulled out by the Protein A column. Furthermore, proteins known to be involved in courtship, such as Fru, were not identified. While these results are not what were expected, they show that the Protein A column is able to pull out proteins that could potentially interact with Lov. The condition chosen for the immunoprecipitation experiments discussed above may not be ideal for identifying proteins interacting with Lov in courtship. Further experiments are needed to determine if Lov is truly interacting with the proteins identified here. Additionally, more co-immunoprecipitation experiments using different conditions are needed to identify other proteins that may interact with Lov.

## CHAPTER 10: SUMMARY, DISCUSSION, AND FUTURE DIRECTIONS

### *What's lov got to do with it?*

The gene jim lovell (*lov*) was identified in a screen looking for genes involved in gravitaxis. Based on sequence homology, *lov* is predicted to be a transcription factor. It shares high homology with another gene, *fruitless*, which is required for courtship, suggesting that *lov* may be involved in multiple behaviors. Examination of the EYFP reporter gene expression in an enhancer trap line mutant, 91Y revealed that *lov* is expressed in two sensory organs involved in gravitaxis, the wing hinge and the leg chordotonal organ. It is also expressed in tarsus in the foreleg of males only, suggesting that there may be a male-specific transcript of *lov* (Figure 2-2). Examination of the *Lov* expression pattern in embryos has identified functions for *lov* in the development of the embryonic nervous system (Figures 4-10-4-12). It is expressed in subsets in neurons in all three types of sensory cells: External Sensory Organs, Chordotonal Organs, and Multiple Dendritic Cells. *lov* is expressed in subset of neurons after the sensory organs have been formed suggesting that *lov* functions as a neural identifier gene that further specifies the sensory organs into particular roles.

Generation of new mutants has provided additional insight into the function of *lov*. New deletion alleles of *lov* reveal that *lov* has a role in courtship. When *lov* expression is altered in these mutants, there is a reduction in amount of time that males will court females (Figure 6-7). Additionally, many of these mutants demonstrate a non-directed courtship in which they perform different steps of courtship but are not directed towards the courtship target (Figure 6-8). Furthermore, one mutant displays male-male courtship (Figure 6-13). This same mutant also has defects in fertility due to defects in

gametogenesis. Finally, ectopic, over, and under-expression experiments using a series of *lov* transgenes alter courtship in a similar manner to the *lov* mutant alleles (Figures 8-4 to Figure 8-11). These results help to confirm that *lov* has a role in courtship.

What do the individual *lov* mutant alleles tell us about the function of *lov*?

There are presently six mutant alleles of *lov*. These mutants have provided much insight into the function of *lov*; most importantly revealing that *lov* has a function in courtship. One of these alleles is a large insertion element. It is believed that this insertional element is disrupting the ability of the enhancers to regulate *lov* expression in a subset of tissues leading to alterations in behavior. The remaining five of these mutants are deletion mutants of various sizes. The deletions fall into two sets: two deletions are upstream of the 91Y insertion site and the remaining three deletions are downstream (see Figure 3-4 for exact locations). All five deletions delete parts of a particular intron and introns often contain regulatory sequences. Therefore, it is possible that these different deletions are affecting different regulatory elements associated with *lov*. By examining the effects the different mutant alleles have on *lov* expression, development and behavior, we can possibly identify regulatory elements have critical to these individual functions of *lov*.

## 91Y

The first mutant allele identified for *lov* was 91Y. It was identified in a screen looking for genes involved in gravitaxis. This is an insertional mutant which has a P{GawB} transposable element inserted in the first large intron of one transcript and upstream of the transcriptional start site of the three other predicted transcripts (Figure 1-9). Using the enhancer trap feature of the P-element, 91Y was found to disrupt *lov*



expression in three locations in the adult: the wing hinge, leg chordotonal organ, and the tarsus (Figure 2-2). Interestingly, disruption of expression in the tarsus was only limited to the foreleg of males. Behavior analysis of 91Y reveals that it reduces the amount of time males court females (Figure 6-7). It also has a high level of non-directed courtship (Figure 6-8). When other behaviors were tested, taste and vision for the 91Y mutant were comparable to wild type (Figures 7-2 and 7-10). The ability of the 91Y mutant to taste normally was somewhat surprising given that there is altered expression in the tarsus of males. The tarsus contains gustatory receptors that are known to be important in courtship. The ability of the 91Y mutant to respond to low levels of the pheromone cVA was slightly reduced as was its activity level in the presence of another fly as compared to wild-type (Figure 7-8 and 7-12). It is possible that the altered courtship observed in 91Y is due to an inability of the males to smell and/or process pheromones properly and a reduced activity level in the presence of other flies. It would appear then that the regulatory elements disrupted by the presence of the insertion may help to regulate these behaviors, possibly in a sex-specific manner.

lov<sup>66</sup>

lov<sup>66</sup> was identified during the mutagenesis screen as a sterile mutant. The mutation produces viable homozygous adults but those adults do not produce offspring. Molecular characterization revealed that there is an approximately 400 base pair deletion starting from the 91Y insertion and ending 77 base pairs upstream from the transcription start site of two of the smaller transcripts (Figure 3-4). Examination of Lov expression in the embryonic nervous system revealed that there is little difference in Lov expression between lov<sup>66</sup> and wild type (Figure 4-12E & E'). Initial courtship assays revealed that

lov<sup>66</sup> had a severe courtship defect (Figure 6-4). However, when lov<sup>66</sup> was placed into the isogenic background, the courtship levels returned close to wild type levels (Figure 6-7). Interestingly, lov<sup>66</sup> males demonstrated male-male courtship behavior (Figure 6-13). When other behaviors were tested, lov<sup>66</sup> mutants had a reduction in the ability to taste sucrose (Figure 7-2). lov<sup>66</sup> homozygotes were also slightly neutral to cVA rather than attracted to it like wild type (Figure 7-8). lov<sup>66</sup> homozygotes had a higher locomotor activity level than wild type (Figure 7-12). These alterations in behavior may be responsible for the male-male courtship.

Further analysis revealed that lov<sup>66</sup> has defects in spermatogenesis. Young lov<sup>66</sup> males initially produce motile, properly individualized sperm. However, in a small number of lov<sup>66</sup> males, as the males age, the number of functional sperm decreases (Figure 5-3). Additionally, while females can accept and store sperm, they appear to be unable to fertilize eggs with that sperm (Table 5-2) and the eggs which they produce are abnormal. When eggs from lov<sup>66</sup> females, who have been mated to lov<sup>66</sup> males, are examined, a large percentage of the eggs do not hatch and most of those eggs are unfertilized (Figures 5-4 and 5-5). The combination of these effects results in very few offspring produced by lov<sup>66</sup> flies.

The fertility and behavioral defects observed in lov<sup>66</sup> suggests that the region of the gene deleted in this mutant may be important in the identification in proper courtship targets through gustatory and olfactory channels as well may also regulate lov function in gametogenesis.

lov<sup>10</sup>

Like lov<sup>66</sup>, lov<sup>10</sup> has a deletion downstream of the insertion. It is a 16 base pair

deletion that starts 77 base pair downstream from the 91Y insertion site (Figure 3-4). Interestingly, the phenotype of this line is quite different from  $lov^{66}$ . Only the Lov expression pattern in the embryo is similar. Like  $lov^{66}$ , expression of Lov in  $lov^{10}$  is similar to wild type (Figure 4-12B & B'). Unlike  $lov^{66}$ ,  $lov^{10}$  is a fertile line that can produce offspring. Like 91Y, there is a reduction in directed courtship and an increase in non-directed courtship (Figures 6-7 and 6-8).  $lov^{10}$  homozygotes are also neutral to cVA rather than attracted like wild type is (Figure 7-8). In the presence of other males in the activity assay,  $lov^{10}$  homozygotes are less active (Figure 7-12). This suggests that the courtship defect observed in  $lov^{10}$  may be due to defects in processing pheromones and responding to that sensory input.

While the regions of the deletions overlap in  $lov^{10}$  and  $lov^{66}$ , the effects the deletions have are different. The defects observed in  $lov^{10}$  suggest that the regulatory region important for courtship may be a very small region which may be contained in the same region that is removed in  $lov^{66}$ . However, in  $lov^{66}$ , removal of additional regulatory regions may be countering deletion of this regulatory element. Further experiments are needed to determine why the effects of these two mutants are so different.

$lov^{38}$

$lov^{38}$  has a 600 base pair deletion upstream of the 91Y insertion site and retains part the P-element (Figure 3-4). Lov expression on  $lov^{38}$  embryos is similar to the wild type expression pattern (Figure 4-12C & C'). Similar to the other lov mutants, it has a courtship defect. There is a significant decrease in directed courtship and a slight increase in non-directed courtship (Figure 6-7 and 6-8). Like the other lov mutants,  $lov^{38}$  homozygotes are slightly neutral to cVA and have reduced locomotor activity level in the

presence of other males (Figure 7-8).  $lov^{38}$  homozygotes have a significantly higher locomotor activity level than wild type flies (Figure 7-12). Again it would appear that these males have an altered ability in processing pheromone information, leading to defects in courtship.

The region of the gene removed by the deletion in  $lov^{38}$  is in a different location to the others previously discussed. This suggests another possible location for regulatory element important for courtship behaviors. Furthermore,  $lov^{10}$  has several alterations in sequence in this region, raising the possibility that the defects observed in that line are due to alteration upstream of the insertion site rather than downstream. The complementation courtship experiments support this possibility.

$lov^{47}$

$lov^{47}$  has a 1400 base pair deletion upstream of the 91Y insertion site and retains part the P-element (Figure 3-4). This deletion again results in a courtship defect with a reduction in the amount of time a male spends courting a female (Figure 6-7).

Furthermore, this line has the highest level of non-directed courtship (Figure 6-8). Of all the  $lov$  mutants,  $lov^{47}$  has the strongest defects in olfaction (Figures 7-6, 7-7 and 7-8).

This line is slightly repelled by both water and moderate concentration of cVA, whereas the other mutants were neutral to water (similar to wild type) and cVA. Additionally, this line was only slightly repelled by low concentration of benzaldehyde whereas other lines were strongly repelled. Therefore, defects in olfactory may be responsible for the observed courtship defects. Finally,  $lov^{47}$  is the only line which has a reduction in *Lov* expression in the embryonic nervous system (Figure 4-12F & F').

The  $lov^{47}$  deletion removes all of the sequence missing in  $lov^{38}$  and an additional

800 base pairs. The behavior defects in the two lines are similar, although more severe in  $lov^{47}$ . This increase in severity may suggest that another regulatory element has been removed or altered in  $lov^{47}$ . Likewise, only  $lov^{47}$  has a change in the Lov expression pattern in the embryonic nervous system, suggesting that the deletion may remove a regulatory element important for the embryonic expression of lov.

$lov^{65}$

Like  $lov^{10}$ ,  $lov^{65}$  has a 16 base pair deletion that starts 77 base pair downstream from the 91Y insertion site (Figure 3-4) as well as several smaller changes upstream of the insertion site. In the embryonic nervous system, the expression of Lov in  $lov^{65}$  is similar to wild type expression (Figure 4-12D & D').  $lov^{65}$  is a fertile line that can produce offspring, has a reduction in directed courtship and an increase in non-directed courtship (Figure 6-7 and 6-8). Furthermore,  $lov^{65}$  homozygotes are also neutral to cVA rather than attracted like wild type is and in the presence of other males,  $lov^{65}$  homozygotes are less active in locomotor activity assays (Figure 7-8 and 7-12). This suggests that the courtship defect observed in  $lov^{65}$  may be due to defects in processing pheromones and responding to that sensory input.

Like  $lov^{10}$ ,  $lov^{65}$  has defects both upstream and downstream of the P-element insertion site. Therefore alterations in regulatory elements in either location could be responsible for the altered behavior. Courtship analysis of complementation cross with  $lov^{38}$  and the similarity of  $lov^{65}$  to  $lov^{10}$ ,  $lov^{38}$ , and  $lov^{47}$  would suggest that it is alterations upstream of the insertion site are responsible for the changes in behavior.

In conclusion, it would appear that the various mutant alleles of lov disrupt normal courtship, possible through alteration in pheromone detection and/or processing.

The location of the deletions suggests that there are multiple regulatory elements in *lov*. Based on the different alleles, a regulatory element important for gametogenesis is located downstream from the 91Y insertion site. There may also be a regulatory element important for courtship in a nearby region. Additionally, there is likely a regulatory element upstream from the insertion site that is responsible for normal courtship and possibly olfaction.

### Future Directions

The work presented in this thesis has only begun to explore the function of *lov* in fertility and neural development in embryos. It is clear that *lov* has a role in courtship, but exactly how it affects this process remains to be established. A number of experiments are needed to determine its role. First, from the mutant analysis, it is clear that all of the mutants have slight defects in pheromone detection and or processing (Figure 7-12). Using the transgenic pUAST-*lov* lines and the *Valium-lovRNAi* lines, the role of *lov* in olfaction can be addressed. Various GAL4 lines that express in neurons important for olfaction and gustatory can be used to over-express and knockdown *lov*. Observing the effects will provide insight into what may be occurring in the *lov* mutants as well as understanding the normal function of *lov* in olfaction and gustation.

As mentioned earlier, *fru* and *lov* share a high sequence homology and are both believed to be transcription factors. They both also contain a BTB domain which is known to be involved in homo- and heterodimerization. Additionally, they both are involved in courtship. Over-expression of *lov* in neurons expressing the male isoform of *Fru* results in a decrease in courtship (Figure 8-8). Furthermore, when *lov* expression is decreased through the use of RNAi in those same neurons, courtship again is reduced

(Figure 8-11). This suggests that Lov and Fru are acting in the same neurons. However, the nature of this relationship is not clear. Therefore, co-immunoprecipitation may be used to determine if Lov and Fru interact, either directly or indirectly. Immunostaining may also be performed to further explore the relationship between lov and fru. Finally, experiment driving the expression of fru-RNAi using 91Y GAL4 drivers could be performed to study the interaction of lov and fru.

Finally, lov<sup>47</sup> reveals a role for lov in metamorphosis. Under poor food conditions, early stage larvae, 1<sup>st</sup> and 2<sup>nd</sup> instar larvae, will attempt to pupate. Additionally, initial immunostaining has established that lov is expressed in the ring gland, a complex gland that regulates hormonal control of metamorphosis. Additional experiments are underway to examine this possible function of lov and include additional immunostaining as well using both pUAST and lovRNAi with ring gland GAL4 lines to alter lov expression in the ring gland.

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